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STUDIES ON SODIUM BALANCE IN *GAMMARUS DUEBENI* LILLJEBORG AND *G. PULEX PULEX* (L.)

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INTRODUCTION

Osmotic regulation in the gammarids, including *Gammarus duebeni* and *G. pulex*, has been previously investigated by Beadle & Cragg (1940*a*) who studied variations in the blood concentration in relation to the concentration of the external medium. *G. duebeni* behaved as a typical brackish-water crustacean in that the blood concentration in normal sea water was only slightly hyperosmotic to the medium, but became progressively more so as the outside concentration was reduced. Below 250 mM/l. the blood concentration remained substantially constant as the external concentration was further reduced, and one feature which distinguished this animal from some other brackish-water forms was the very low external concentration at which a high blood level could be maintained. *G. pulex*, on the other hand, behaved in a manner similar to many fresh-water animals in that the blood concentration rose when the external concentration was increased above the level found in fresh water, and when this exceeded the normal blood concentration the animals were no longer able to survive. Kinne (1952) confirmed these results on *G. duebeni* and, in addition, observed that the animals could be adapted to live in fresh water (Kiel tap water) if the external concentration was gradually reduced. Records of the distribution of *G. duebeni* also indicate the ability of this species to live in fresh water: thus Reid (1939) showed that *G. duebeni* is the dominant fresh-water gammarid in Ireland and Hynes (1954) describes the distribution of the species in fresh-water locations on the West Coast of England and Wales.

Since it has been shown that both in the laboratory and in the field *G. duebeni* is capable of living in fresh water, it is of great interest to see if the osmoregulatory mechanism displayed by the animal when in fresh water is similar to that of the truly fresh-water species, *G. pulex*, or if there are differences which may throw light on the problem of the adaptation of brackish-water animals to fresh water.

Beadle & Cragg (1940*b*) attempted to demonstrate differences by the behaviour of the two species in static distilled water (i.e. in volumes of distilled water changed at intervals). They reported that *G. pulex* survived much longer than *G. duebeni* and that by the end of the experiment the blood concentration of the former was only slightly reduced. They also found that specimens of *G. duebeni* from a fresh-water locality survived in distilled water longer than animals from brackish water, although more extensive experiments of the same kind by Hynes (1954) failed to confirm this.

However, later experiments with *G. pulex* (Beadle & Shaw, unpublished) using continuously running distilled water showed that the animals survived for less than 2 days, and during this time the blood concentration was greatly reduced, being rapidly restored again when the animals were returned to tap water. This pointed to the importance of ion uptake mechanisms in maintaining the normal blood concentration even from the very low concentrations which build up in the static distilled water experiments. This is contrary to the conclusion reached by Beadle & Cragg (1940*b*), who maintained that ion uptake mechanisms were of little importance in the gammarids, although it does not invalidate their suggestion that salt retention may form an important part of the osmoregulatory mechanism. Beadle (1943) suggested that the main factors involved in salt retention were the low permeability of the body surface and the production of a dilute urine, although the active control of salt loss was also envisaged.

The maintenance of the normal blood concentration must result from a balance between the rate of loss of salts, as determined by the factors involved in salt retention, and the rate of active uptake of salts. If these two rates can be measured it is then possible to assess the balance conditions and, hence, the osmoregulatory ability of an animal in low external concentrations can be accurately determined.

Balance conditions, determined in this way, have been reported previously for *Astacus pallipes* (Shaw, 1959*a*) and essentially the same procedure has been adopted to study sodium balance in the two *Gammarus* species. In this paper special attention is paid to a comparison of the properties of the sodium absorbing mechanisms in the two species. Loss rates are measured quantitatively without attempting to evaluate the importance of different factors involved in salt retention.

MATERIALS AND METHODS

Gammarus duebeni was collected from Meggies Burn, a small brackish-water stream in Northumberland, and this is the same locality from which Beadle & Cragg had obtained their specimens. On occasions when the salinity of the stream water was measured it was between 40 and 50 % of that of sea water. The animals were maintained in the laboratory in 2 % sea water and fed on dead leaves. *G. pulex pulex* was collected from a fresh-water pond at Monkton, Co. Durham (sodium concentration of the water = 0·6 mM/l.) and kept in the laboratory in a 0·3 mM/l. NaCl solution.

In describing the quantitative measurements of rates of uptake and loss of sodium the same terminology has been used as in the previous study of sodium balance in *Astacus pallipes* (Shaw, 1959*a*).

Sodium influx was measured by use of the sodium isotope, ^{24}Na. The tracer was added to the external solution and the radioactivity of the solution recorded continuously in an apparatus similar to that used for *Astacus* (Shaw, 1960). A diagram of the apparatus is shown in Fig. 1. The Perspex animal chamber contained about 10 ml. of water which was circulated through a flow-type Geiger counter (Twentieth-century Electronics, type FW 10). The isotope was introduced into the water with

sufficient non-active sodium chloride to give the desired sodium concentration, and the radioactivity of the solution was continuously recorded by means of a recording ratemeter. For an influx measurement at an external concentration below 1 mM/l. about ten adult specimens (each weighing about 40 mg.) were introduced into the animal chamber. For higher external concentrations the number of animals was increased up to a maximum of thirty in order to maintain conditions where the total internal sodium greatly exceeded the external sodium. The influx was calculated as before (Shaw, 1959*a*). The sodium concentrations of the external solution and of the blood were measured by means of an EEL flame photometer after appropriate dilution of the sample.

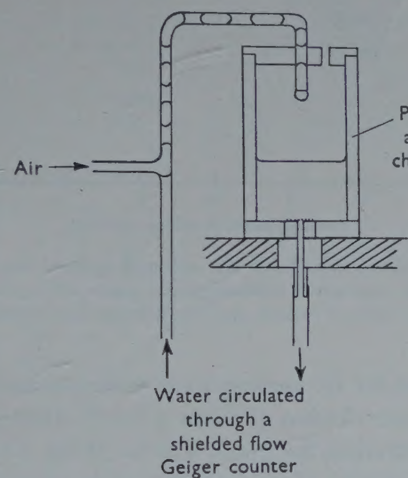

Fig. 1. The apparatus for the measurement of sodium influx.

RESULTS

Gammarus duebeni

(*a*) *Sodium influx*

Measurements of sodium influx at different external concentrations for animals from 2% sea water are shown in Fig. 2. The relation between the influx and the external concentration is non-linear and resembles, in certain aspects, that found in *Astacus* (Shaw; 1959*a*, 1960). As the external concentration is increased the influx tends towards a maximum value, indicating that the inward transport of sodium is effected by a rate-limited process. In *Astacus* it was shown that this relationship could be approximated by the Michaelis equation, influx $= K\{C/(K_m+C)\}$, where K is the maximum rate of transport, C the external concentration, and K_m the external concentration at which half the maximum influx is reached. In *Astacus* the value of K_m was between 0·2 and 0·3 mM/l. In *Gammarus duebeni* the same equation can be used: in Fig. 2 the dotted line represents the curve, influx $= 0{\cdot}95\{C/(1{\cdot}5+C)\}$. An important distinction from *Astacus* may be noted in that the value of K_m (1·5 mM/l.) for *G. duebeni* is about six times greater than that found for the crayfish. This points to the fact that the sodium transporting

system in *Astacus* has a much higher affinity for sodium ions than the corresponding system in *G. duebeni*. It is shown later (p. 10) that *G. pulex* has a transporting system very similar to *Astacus* and that the distinction between the two systems (i.e. between that of *G. duebeni* and *pulex*) is of considerable functional importance.

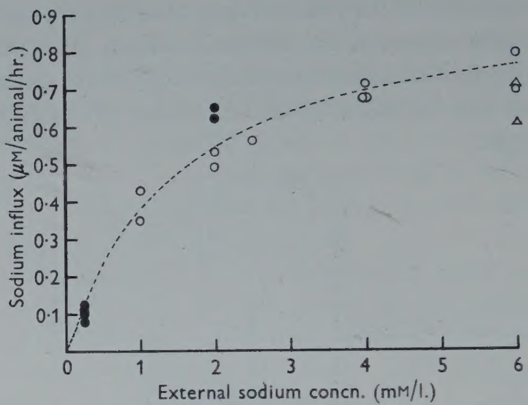

Fig. 2. The relation between sodium influx and the external sodium concentration in *G. duebeni* adapted to 2 % sea water. ●, represents measurements made with groups of ten animals in the animal chamber; ○, with groups of twenty and △, with groups of thirty.

The influx measurements for *G. duebeni* were made on animals adapted to 2 % sea water with a sodium concentration of about 9 mM/l. It can be seen from Fig. 2 that at this external concentration the influx will be about 0·8 μM/hr. Now if the tracer influx gives a true measure of the rate of uptake of sodium by the animals then, since they were in a steady state at this external concentration, the uptake should be balanced by an equal rate of loss of sodium. Direct measurements of the rate of loss of sodium from animals adapted to 2 % sea water confirm the validity of the tracer technique under these conditions.

(*b*) *Sodium loss rate*

Sodium loss rate was measured by the rate of increase in sodium concentration of a volume of deionized water into which the animals were placed. For each experiment ten animals were placed in 50 ml. of water and the sodium concentration was measured at intervals over a period of 1–2 hr. During this period the rate of increase of sodium concentration was linear, so that the rate of loss of sodium from the animals could be easily calculated. The results of these measurements are shown in Table 1. The mean rate of loss was 0·76 μM/hr./animal and this was in reasonable agreement with the expected value (0·8 μM) from the influx measurements.

The validity of the tracer measurements is further demonstrated by measurement of the net loss of sodium into solutions of sodium chloride with a concentration lower than 9 mM/l. and from which the sodium influx was already known by previous measurements (see Fig. 2). The results are shown in Fig. 3 where at three different external concentrations (1, 2 and 3 mM/l.) the sum of the net sodium loss

rate and the influx is approximately equal to the total loss rate in the absence of sodium influx (i.e. the loss rate into deionized water).

In addition, these experiments show that animals adapted to 2 % sea water are not in balance at lower concentrations, since there is always a net sodium loss.

Table 1. *The rate of sodium loss in* Gammarus duebeni *from 2 % sea water*

Group no.	Sodium loss rate (μM/hr./animal)
1	0·75
2	0·81
2	0·67
4	0·74
5	0·84
Mean	0·76
S.D.	±0·07

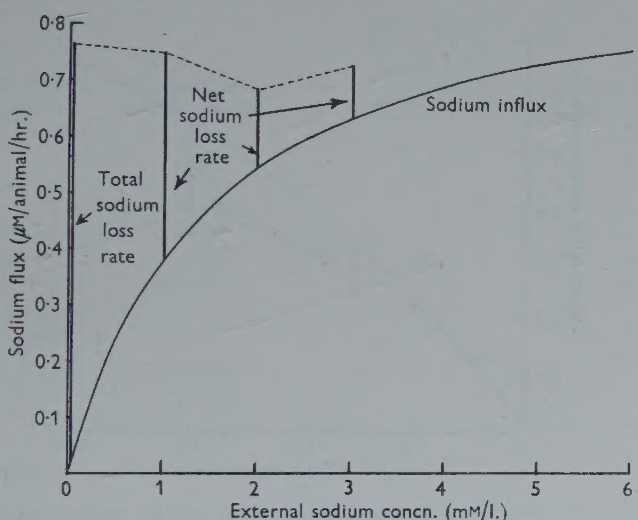

Fig. 3. The net rate of sodium loss at different external concentrations in *G. duebeni* adapted to 2 % sea water. The net loss is indicated by the vertical lines. The influx is represented by the smoothed curve from Fig. 2. The dotted line represents the calculated total loss rate.

(*c*) *Adaptations to low concentration*

Despite the fact that in short-term experiments with animals from 2 % sea water, sodium balance cannot be maintained at lower external concentrations, the animals may be gradually adapted to these concentrations. Thus in the laboratory, animals were successfully adapted to very dilute sea water and to sodium-chloride solutions with a sodium concentration of 2 mM/l. and often with a concentration as low as 1 mM/l. A progressively smaller percentage of the animals survived in lower concentrations of sodium chloride and the limit appeared to lie between 0·2 and 0·3 mM/l.

Clearly adaptation to these low concentrations must involve a change in the balance conditions, and must be effected either by an increase in the influx or a decrease in the loss rate, or by a combination of both. These rates were therefore measured in a group of animals which had been successfully adapted to an external sodium concentration of 0·25 mM/l. Measurements of sodium influx and the net sodium movement were made within a range of external concentrations from 0 to 2 mM/l. The results are shown in Fig. 4. Balance was maintained at the concentration to which the animals were adapted (0·25 mM/l.). At higher concentrations (at 0·75 and 1·5 mM/l.) a net uptake of sodium took place, whereas in animals adapted to 2 % sea water a net loss occurred at these concentrations. The loss rate (calculated from the difference between the influx and the net sodium movement—represented in Fig. 4 by the dotted line) appeared to increase with increasing external concentration. This was also observed in *Astacus* (Shaw, 1959*a*) and is probably due to an exchange diffusion component in the influx at high net uptake rates.

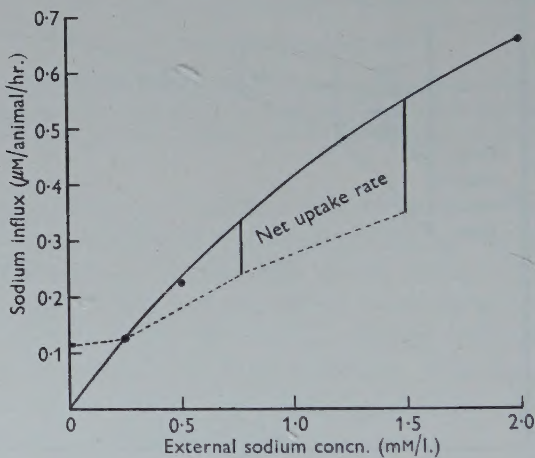

Fig. 4. The relation between the influx and the external sodium concentration, together with measurements of the net sodium uptake in a group of *G. duebeni* adapted to 0·25 mM/l. NaCl.

The balance conditions were clearly different from those found in the animals from 2 % sea water and it was important to determine whether this was due to changes in the influx or in the loss rate. The effect of adaptation to a low external concentration on the influx was first considered. In one series of experiments the influx at an external concentration of 0·25 mM/l. was measured for a group of animals adapted to 2 % sea water. The group was then adapted to 0·25 mM/l. sodium chloride and the measurement repeated. This was successful in a few cases: in most, however, some of the animals died during the course of adaptation. In the other series of experiments a large group of animals were adapted to the low external concentration and then smaller groups taken from those which had survived. This method had the advantage that many animals were available for the experiment, but introduced an element of selection into the comparison. The results of both series

of experiments are shown in Table 2. A comparison of the mean influx in animals from 2 % sea water and from 0·25 mM/l. NaCl shows that the adaptation has involved an increase in the influx of only about 25 %. In the case of the adapted animals selected from a large group the increase in the influx was greater (about 65 %). However, it cannot be argued that any individual animal had increased its influx by this amount, since the technique may simply have selected out those animals which had a high influx initially. In any event it is clear that the influx level found in the animals adapted to 0·25 Mm/l. NaCl is quite insufficient to balance the loss rate, if this was maintained at the same level as it was in the animals from 2 % sea water. Thus an increase in the influx is not a major part of the mechanism of adaptation to the lower concentrations.

Table 2. *The effect of adaptation to low external concentrations on the sodium influx in* Gammarus duebeni

Groups adapted to 2 % sea water	Sodium influx (μM/hr./animal)	Active animals selected from a large group adapted to 0·25 mM/l. NaCl	Sodium influx (μM/hr./animal)
1	0·116	1	0·163
2	0·096	2	0·146
3	0·110	3	0·146
4	0·08	4	0·146
6	0·118	5	0·231
Mean	0·102	Mean	0·166
S.D.	±0·015	S.D.	±0·037
Groups adapted to 0·25 mM/l. NaCl			
1	0·126		
2	0·118		
3	0·118		
4	0·143		
Mean	0·126		

The effect of adaptation to a low external concentration on the rate of loss can now be considered. The rate of loss of sodium from animals adapted to 2 mM/l. NaCl and to 0·25 mM/l. NaCl was measured in the same manner as for animals from 2 % sea water. The results are shown in Table 3. The reduction in the rate of loss is striking. In animals adapted to 2 mM/l. NaCl the loss rate is one-half of that of animals from 2 % sea water and in the animals from 0·25 mM/l. NaCl it is halved again. This is clearly the most important factor in the adaptation of the animals to the low external concentrations.

The mean rate of loss for animals adapted to 0·25 mM/l. NaCl was 0·17 μM/hr., and this was somewhat higher than the measured influx at this concentration found in animals adapted to 2 % sea water (Table 2). Thus it would appear that only those animals with a relatively high influx in 0·25 mM/l. NaCl will be able to achieve balance at this concentration and this would seem an adequate explanation of why only a relatively small proportion of the animals survive the adaptation.

Table 3. *The effect of adaptation to low external concentrations on the rate of sodium loss in* Gammarus duebeni

Groups adapted to 2 % sea water	Sodium loss rate (μM/hr./animal)	Groups adapted to 0·25 mM/l. NaCl	Sodium loss rate (μM/hr./animal)
Mean value from Table 1	0·76±0·07	1	0·14
		2	0·13
Groups adapted to 2 mM/l. NaCl		3	0·13
1	0·23	4	0·18
2	0·58	5	0·14
3	0·42	6	0·20
4	0·37	7	0·21
5	0·46	8	0·20
6	0·24	9	0·16
		10	0·22
Mean	0·38	Mean	0·17
S.D.	±0·13	S.D.	±0·03

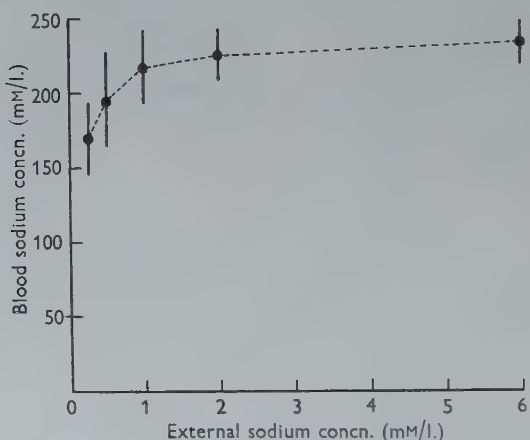

Fig. 5. The relation between the sodium concentration of the blood and the external solution in *G. duebeni*

(*d*) *Blood sodium concentration*

It is reasonable to suppose that the rate of loss of sodium is directly proportional to the blood concentration. The level of sodium in the blood is thus a factor concerned in determining the loss rate, and the reduction in the loss rate when the animals are adapted to low external concentrations could follow from large reductions in the blood concentration. Measurements of blood sodium were therefore made on animals which had survived adaptation to various low external concentrations. The results are shown in Fig. 5. A decrease in the external concentration from 6 to 1 mM/l. brought about only a slight change in the blood concentration. At external concentrations below 1 mM/l. there was a steep fall in blood concentration, although at the lowest concentration in which the animals would survive the blood concentration was still nearly 75 % of its initial value. Hence a reduction in the blood concentration is not directly responsible for the decrease in loss rate. It

follows, therefore, that the reduction in loss rate must result from either a decrease in the permeability of the body surface or a reduction in the loss of salts through the urine, or a combination of both. It is difficult to decide between these possibilities. It is probable that *G. duebeni* can produce a dilute urine, since the antennal gland is morphologically similar to that of *G. pulex* and in both it differs from that of a marine species such as *G. locusta* (Schwabe, 1933; Hynes, 1954). But it does not follow that this will allow an effective regulation over the loss rate. Even if the urine could be varied between a very dilute and an iso-osmotic solution it would require a very rapid rate of production to produce large changes in the loss rate. Thus if animals from 2% sea water produced an iso-osmotic urine and animals from 0·25 mM/l. NaCl produced a very dilute urine, and if the difference in loss rate between the two (i.e. 0·59 μM/hr./animal—from Table 3) is brought about solely by the difference in urine concentration, then the urine must be produced at a rate equivalent to about 7·4% of the body weight per hour. This is a fast rate but not an impossible one. If measurements of urine production rate ultimately show that this rate is not achieved, then one must postulate a mechanism by which the animal can regulate its surface permeability.

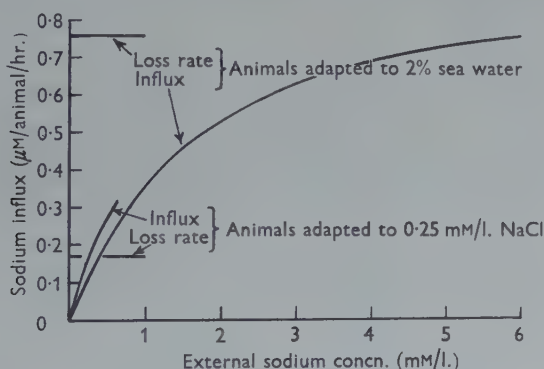

Fig. 6. A summary of the balance conditions in *G. duebeni*. The curves represent the influx for animals adapted to 2% sea water and to 0·25 mM/l. NaCl and the horizontal lines represent the loss rates under these conditions.

(*e*) *Summary of balance conditions*

The results of the influx and loss rate measurements on *G. duebeni* are summarized in Fig. 6. This shows that in 0·25 mM/l. NaCl only those animals with an influx above average are able to maintain balance. On the other hand, in 0·5 mM/l. NaCl the majority of animals will maintain balance, although some individuals with a low influx or high loss rate may not. At an external sodium concentration of 1 mM/l. balance can always be achieved, and there is some margin of safety. These conclusions are borne out by the survival of the animals during adaptation and by the measurements of the blood concentration. At external concentrations down to 1 mM/l. survival is good and the blood concentration is maintained. Below this the survival rate becomes progressively poorer and the blood concentration falls rapidly.

Gammarus pulex pulex

(*a*) *Sodium influx*

Measurements of sodium influx were made over a range of external concentrations from 0·05 to 2 mM/l. NaCl in animals which had been previously adapted to 0·1 mM/l. NaCl for several days. The specimens were approximately the same weight and size as the specimens of *G. duebeni* and therefore the influx values could be directly compared. The results are shown in Fig. 7. The relation between the

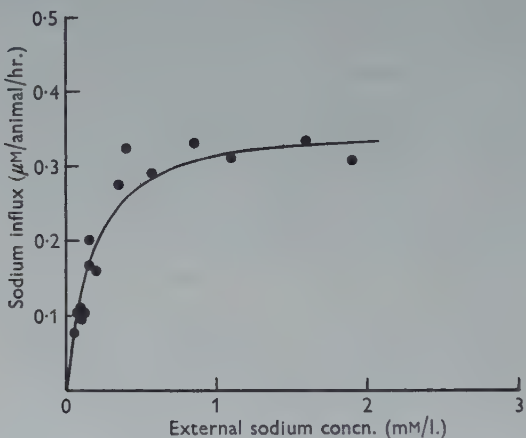

Fig. 7. The relation between the influx and the external sodium concentration in *G. pulex* adapted to 0·1 mM/l. NaCl.

influx and the external concentration has the same general form as that found in *Astacus* (Shaw, 1959*a*) and in *G. duebeni*. However, there are important differences between the relationship as shown in the two *Gammarus* species which may be listed as follows: (*a*) the maximum influx for *G. pulex* is less than half that for *G. duebeni*, (*b*) the maximum influx for *G. pulex* is reached at a much lower external concentration than for *G. duebeni*, and (*c*) the value of K_m for *G. pulex* (0·15 mM/l.) is much lower than the corresponding value (1·5 mM/l.) for *G. duebeni*. This points to the fact that although the maximum rate of sodium transport is lower in *G. pulex* than in *G. duebeni*, the transporting system in *G. pulex* has a much higher affinity for sodium ions. It follows, therefore, that at the lower external concentrations the uptake rate for *G. pulex* may exceed that for *G. duebeni*. The importance of this fact for the maintenance of sodium balance at low external concentrations is discussed below. The relation between the influx and the external concentration as found for *G. pulex* bears a much closer resemblance to that of *Astacus*, and it is suggested that the high affinity of the transporting system for sodium ions is a special feature of truly fresh-water forms.

(*b*) *Survival in low external concentrations*

Gammarus pulex surpasses *G. duebeni* in its ability to survive in low external concentrations, as might be expected from the normal fresh-water habit of the species. Thus *G. pulex* can be readily adapted to sodium chloride solutions with a concentration as low as 0·1 mM/l. and the percentage survival is high. It has been shown that *Astacus pallipes* (Shaw, 1959*a*) and *Potamon niloticus* (Shaw, 1959*b*) could be characterized by the minimum external sodium concentration at which sodium balance could be maintained. A similar situation was found for *G. pulex*. Groups of ten animals were placed in a limited volume (50 ml.) of deionized water and the external concentration allowed to rise until a steady state was reached. The water was then replaced and the procedure repeated until the lowest external concentration at which balance could be maintained was reached. The results of these experiments are shown in Table 4. The mean value was 0·06 mM/l. It may be noted that this value is very similar to that found for *Astacus* (0·04 mM/l.; Shaw, 1959*a*) and for *Potamon* (0·05 mM/l.; Shaw, 1959*b*). This external concentration is sufficiently low to allow the animals to survive in all normal fresh waters found in this country. It is probable that a value around 0·05 mM/l. is characteristic of the true fresh-water Crustacea.

Table 4. *The minimum external sodium concentration for balance in* Gammarus pulex

Group no.	External sodium concentration (mM/l.)
1	0·06
2	0·055
3	0·06
4	0·07
5	0·05
6	0·065
Mean	0·06

G. pulex is thus able to survive in concentrations well below the limit for *G. duebeni*. It is interesting that the limiting concentration for *G. duebeni* is similar to that found for the brackish-water crab, *Eriocheir sinensis* (0·2–0·5 mM/l.; Krogh, 1939; Koch & Evans, 1956). *G. duebeni* resembles this crab in other physiological features too. For example, they both maintain a high blood concentration and are able to survive in full-strength sea water—features which distinguish them from many true fresh-water animals.

(*c*) *Balance conditions at different external concentrations*

At any given external concentration balance results from the equality between the rates of sodium uptake and loss, both of which may be variable. In *Astacus*, adaptation to the lower concentrations is brought about largely by an increase in the uptake rate whereas in *G. duebeni*, at concentrations below 2% sea water, this is

Table 5. *The effect of adaptation to low external concentrations on the sodium influx and the rate of sodium loss in* Gammarus pulex

Groups adapted to	Sodium influx (μM/hr.) From 0·3 mM/l. NaCl	Sodium influx (μM/hr.) From 0·06 mM/l. NaCl	Sodium loss rate (μM/hr.)
0·3 mM/l. NaCl	0·16	—	0·18
0·06 mM/l. NaCl	0·25	—	0·12
0·06 mM/l. NaCl after further sodium loss	—	0·084	0·09

achieved largely by a reduction in the loss rate (p. 7). In *G. pulex* both features were found to be important. Measurements were made of the influx and loss rate in animals adapted to 0·3 mM/l. NaCl and to their minimum equilibrium concentration (0·06 mM/l.). The results are given in Table 5, which shows the influx and loss rate measurements on three groups of animals. The first was adapted to 0·3 mM/l. NaCl, the second to 0·06 mM/l. and the third adapted to 0·06 mM/l. and then subjected to further sodium loss. In the third group the blood concentration was reduced below that of the first two groups, but still required an external concentration of 0·06 mM/l. to maintain balance. It can be seen that where the influx was measured at the balance concentration (groups 1, 3) there was good agreement between the influx and the loss rate. Where the influx was measured at the same external concentration (groups 1, 2) it can be seen that adaptation to the lower concentration had involved an increase in the influx by a factor of 1·5 and a reduction in the loss rate by a similar amount. Additional sodium loss (group 3) led to a further reduction in the loss rate.

The increase in the influx must have been brought about by activation of the transporting system, but the reduction in loss rate might have been due simply to a fall in blood concentration. Therefore measurements of the blood sodium concentration were made on groups of animals which had been similarly treated, and the results are shown in Table 6. The fall in blood concentration was not sufficient to account for the reduction in loss rate so that, as in *G. duebeni*, some other explanation must be sought to account for this phenomenon. Again this might be due to regulation of the urine concentration, but a decision on this must await measurements of urine production rate.

Table 6. *The effect of adaptation to low external concentrations on the blood sodium concentration in* Gammarus pulex

Groups adapted to	Blood sodium concentration (mM/l.)	S.D. ±mM/l.	n
0·3 mM/l. NaCl	127	16	9
0·06 mM/l. NaCl	113	18·5	8
0·06 mM/l. NaCl after further sodium loss	105	7	8

DISCUSSION

The work of Beadle & Cragg (1940*a*) and Kinne (1952) showed that *G. duebeni* behaves as a typical brackish-water crustacean which can maintain its blood concentration at low external concentrations. It has now been shown that it owes its ability to survive in these low concentrations to the fact that it can reduce the rate of salt loss under these conditions. This ability has allowed the species to extend into at least part of the fresh-water range, although it is still unable to compete with *G. pulex* in its ability to survive in concentrations below 0·5 mM/l. It remains to be discovered whether the mechanism of the reduction of loss rate can be explained solely in terms of the ability to produce a dilute urine, or whether an active mechanism for controlling surface permeability must be looked for.

When *G. duebeni* is adapted to low concentrations the rate of salt loss is not very different from that of *G. pulex*, so that the superiority of the latter species in surviving in low concentrations clearly lies in the special properties of its sodium uptake mechanism. In both species sodium uptake plays a vital role in the maintenance of sodium balance; in the absence of this mechanism sodium is lost from both at a rate which would at least halve the total body sodium in the course of a single day. The sodium uptake mechanisms of *G. pulex* bears many resemblances to that found in *Astacus pallipes*, particularly in that (*a*) the uptake mechanism has a very high affinity for sodium ions, and the maximum transport rate is reached at an external concentration of about 1 mM/l.; and (*b*) the rate of uptake is increased by a relatively small drop in the internal sodium content. Apart from the ability of *G. pulex* to reduce the rate of loss in response to a lowered blood concentration (a fact which was not demonstrated in *Astacus*) the mechanisms of sodium balance in the two species have much in common. These features may be characteristic of fresh-water crustaceans in general.

The mechanism of sodium uptake in *G. duebeni*, although basically similar to that of *G. pulex*, shows several important differences upon which the ability to maintain balance at low external concentrations can be seen to depend. The mechanism in *G. duebeni* has a relatively low affinity for sodium ions and, although the maximum transport rate is greater than in *G. pulex*, this rate is not reached until the external concentration is about 10 mM/l. Further, a relatively large fall in blood concentration below 200 mM/l. has little or no effect on increasing the uptake rate.

The importance of the distinction between the two types of mechanism displayed by *G. duebeni* and *G. pulex* can be seen by reference to Fig. 8, where the curves relating influx to external concentration in the two species are compared. It can be seen that whereas above 1 mM/l. the influx in *G. duebeni* may greatly exceed that in *G. pulex*, at external concentrations below this the situation is reversed. At 0·1 mM/l., for example, the influx in *G. pulex* is nearly three times greater than in *G. duebeni*. Moreover, on decreasing the external concentration from 2 to 0·1 mM/l., in *G. pulex* the influx only drops to 40% of its value, whereas in *G. duebeni* it falls to as low as 10%. It is therefore suggested that the high affinity for sodium

displayed by the uptake mechanism of *G. pulex* is an important adaptation to survival in low external concentrations.

The problem of the occurrence of populations of *G. duebeni* in apparently true fresh-water habitats remains to be settled. It must be emphasized that the specimens of *G. duebeni* used in this work were derived from a permanently brackish-water locality, and it has been suggested previously (Beadle & Cragg, 1940*b*) that the animals from fresh-water habitats constitute a physiological variety of the species. This view was questioned by Hynes (1954) who failed to find differences in survival time between animals derived for brackish and fresh water when these were exposed to distilled water. However, it is felt that the possibility could well be re-examined now that more sensitive methods are available. It may be that the fresh-water forms have evolved an uptake mechanism which is closer to the *G. pulex* type. An examination of these animals holds promise of some interesting eco-physiological problems.

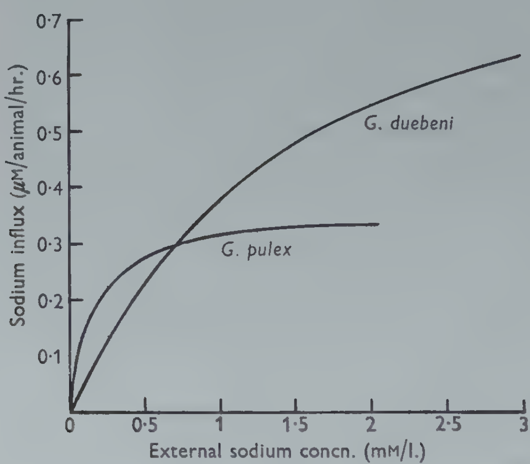

Fig. 8. A comparison between the influx/external concentration curves for *G. duebeni* and *G. pulex*.

SUMMARY

1. The mechanisms of sodium balance in *Gammarus duebeni* and *G. pulex*, adapted to various external concentrations, were compared.

2. *G. duebeni* could be adapted to live in 1 mM/l. NaCl solution and, in some cases, to concentrations down to 0·2 mM/l. *G. pulex* could survive in concentrations as low as 0·06 mM/l.

3. The sodium loss rate in *G. duebeni* adapted to 2 % sea water was much higher than in *G. pulex* but was reduced to about the same level when the animals were adapted to low external concentrations.

4. In both species there was a non-linear relationship between sodium influx and the external sodium concentration. In *G. duebeni* the uptake mechanism was saturated at an external concentration of about 10 mM/l., whereas in *G. pulex*

saturation was reached at a much lower concentration. The maximum rate of uptake was greater in *G. duebeni* than in *G. pulex.*

5. In both species adaptation to low concentrations involved a small increase in the sodium influx and a reduction in the loss rate.

6. The most important factor in the superiority of *G. pulex* over *G. duebeni* in surviving at low external concentrations is the high affinity for sodium displayed by the uptake mechanism in *G. pulex.*

We should like to thank Dr H. B. N. Hynes who kindly checked the identification of our specimens. Part of the expenses of this work were defrayed by a grant from the Royal Society. During the course of this work one of us (D.W.S.) was in receipt of a D.S.I.R. Research Fellowship.

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THE RESPONSE OF A HYDROID TO WEAK WATER-BORNE DISTURBANCES

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INTRODUCTION

The ability to detect moving objects in an aqueous medium by the mechanical disturbance they produce is known for a number of animal groups; leeches (Whitman, 1898; Herter, 1929), amphibians (Whitman, 1898; Kramer, 1933), fish (see recent review by Lowenstein, 1957), and even amoebae (Schaeffer, 1916). Most coelenterates, however, appear able to respond to the presence of a predator or to prey only when such objects actually touch some part of their surface. This is probably true even for those forms such as *Gonionemus* and *Corymorpha* which behave as though they were actively seeking food (Yerkes, 1902; Torrey, 1904). Although hydroid medusae are able to locate accurately a tactually stimulated portion of their subumbrella surface with their manubrium (Romanes, 1885; Horridge, 1955), there is no evidence that they are able to react to objects at some distance. Hardy (1958), however, described capture of *Balanus* nauplii by 'accurate deft twists of the manubrium' in a scyphozoan ephyra and suggests that vibration perception by statocysts may be involved. Hydroid polyps have been shown to be able to bend toward sources of chemicals diffusing in the water (Loomis, 1955), but this response probably occurs normally only following capture of a suitable prey. The great sensitivity of *Calliactis* to movement above the oral disk (Passano & Pantin, 1955) suggests that this species might react to the proximity of moving animals.

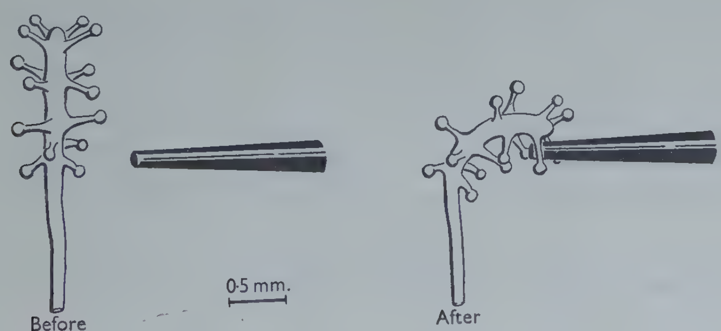

Fig. 1. A *Syncoryne* polyp before and after lightly tapping the manipulator holding the glass rod shown entering from the right.

It was observed that polyps of the hydroid *Syncoryne mirabilis* would bend toward a vibrating glass rod placed in their vicinity in a dish of sea water (Fig. 1). This was most easily shown by mounting a fine-tipped rod in a manipulator and, after moving the tip near a polyp, lightly tapping the manipulator. The polyp would immediately bend toward the rod unless it were placed directly above the mouth of a polyp, in which case the polyp would respond by contracting.

The present paper describes some of the characteristics of stimuli effective in evoking this response, gives evidence that such polyp bending plays an important role in the life of the animal, and attempts to explain the mechanisms of detection of stimuli and determination of the direction of bending.

METHODS

Colonies of *Syncoryne* were maintained in running sea water at approximately 16° C. *Syncoryne* polyps are about 1·5 mm. in length. Individual polyps were removed from a colony and pinned by the stalk to a piece of cork mounted on the bottom of a dish of sea water. All such dishes were kept in running sea water until used in an experiment and were returned to this sea water whenever their temperature rose much above 16° C. In experiments involving ablation of tentacles, one-fourth of the sea water in the dish was replaced by isotonic magnesium chloride and the polyps were left in this solution until they no longer responded to touch. After removal of the desired parts, the dishes were restored to running sea water until the animals recovered their sensitivity.

Controlled stimuli were given to the polyps by means of a prodder attached to a speaker cone driven electrically by square pulses from a Grass S4 stimulator. The prodder consisted of a glass rod with a 1 cm. piece of cover-slip cemented to its end, perpendicular to the direction of prodder movement. The speaker and prodder were mounted in a manipulator. In an experiment the prodder was positioned with the cover-slip parallel to the long axis of a hydroid polyp. It was hoped that by using a vibrating area many times larger than the polyp, differences between experiments due to slightly different lateral placement of the prodder would be minimized.

The prodder movement was calibrated by putting the flat surface under a compound microscope and measuring its movement with an ocular micrometer when the speaker was energized by various d.c. voltages. The displacement of the prodder was a linear function of the applied voltage in the range of strengths used in the experiments. As the usual input to the speaker was not a constant current, but a brief pulse (most often 5 msec. in duration), and since the prodder was placed in sea water which would impede its movement, it is felt that the displacement in air as measured by the microscope represented the maximum possible excursion of the prodder.

In all experiments the polyp response measured was a bending of the hydranth. In some cases one tentacle, or a few, alone twitched toward the source of stimulus without movement of the whole polyp. This response was most often seen when using a prodder with a fine tip; when using a prodder with a large surface, the bending response of the whole polyp almost always had a lower threshold than the

individual tentacle twitches, and the latter could, for the most part, be ignored. Occasionally one or more polyps in a dish would show continued spontaneous tentacle twitching. Examination of such polyps always revealed the presence of a hypotrichous ciliate crawling about their surface. This indicates that small local disturbances are sufficient to cause polyp reactions.

Responses were best seen in hydroids which had been held in the laboratory for several days. Possibly due to the disturbances involved, freshly collected polyps were relatively unreactive. Once in the laboratory, polyps would show responsiveness for several weeks, even though they were not intentionally fed and the average polyp size decreased greatly.

RESULTS

Characteristics of the effective stimulus

(*a*) *Directionality*

By using stimuli caused by a stepwise increase or decrease in the current energizing the speaker, it was shown that the polyp response did not depend upon the direction of polyp displacement; a sudden advance of the prodder or a sudden retreat were equally able to evoke bending of the polyp towards the prodder. A square-wave input to the prodder system was somewhat more effective than a step increase or decrease (about 20% less voltage required) if the duration of the pulse was 5 msec. or more. At durations less than 5 msec., the threshold voltage to square pulses increased with decreasing duration. This was probably due to mechanical damping of the stimulating system.

The most obvious demonstration of the independence of the bending response on the direction of polyp displacement was given by an experiment in which the prodder was placed between two polyps growing from a common branch. Here the directionality of the stimuli delivered to each polyp was exactly reversed; whenever the prodder moved toward one polyp it moved away from the other. Even in this case, however, both polyps bent toward the prodder when the stimulus exceeded threshold.

(*b*) *Distance*

In a number of trials, each involving many measurements on a single polyp, the strength of stimulus required to cause polyp bending was found to be an exponential function of the distance of the prodder from the polyp (Fig. 2). This indicates a drop in the stimulus strength with increasing distance from the prodder. The threshold intensity at different distances may be in part affected by greater damping of the prodder on larger excursions, and the intensity-distance curve may also be influenced by 'focusing' of the disturbance by the large flat prodder surface. Noteworthy was the sensitivity of the polyps. At a distance of 0·5 mm. from the centre of a polyp, responses were obtained with a prodder displacement of from 2·5 to 3 μ.

(*c*) *Frequency*

Because *Syncoryne* will respond to a single excursion of the prodder, bending toward a prodder vibrating at any frequency does not mean that the polyp is

sensitive to this frequency. The polyp may only be responding to the first pulse in the series.

To test the effect of frequency on the response, a polyp was stimulated by a prodder vibrating at a given frequency, while the amplitude of the pulsations was increased until the polyp responded. The rate of amplitude increase was 20 % per pulse at frequencies less than one per second, and 20 % per second at frequencies greater than this. This method gave the surprising result that animals would not respond at all to frequencies greater than five per second. The average maximum frequency which would cause polyp bending was three per second (range 1–5 per second, fourteen trials on six different polyps). The threshold intensity for bending remained rather constant at frequencies from one per 10 sec. to the maximum frequency to which the polyp would respond.

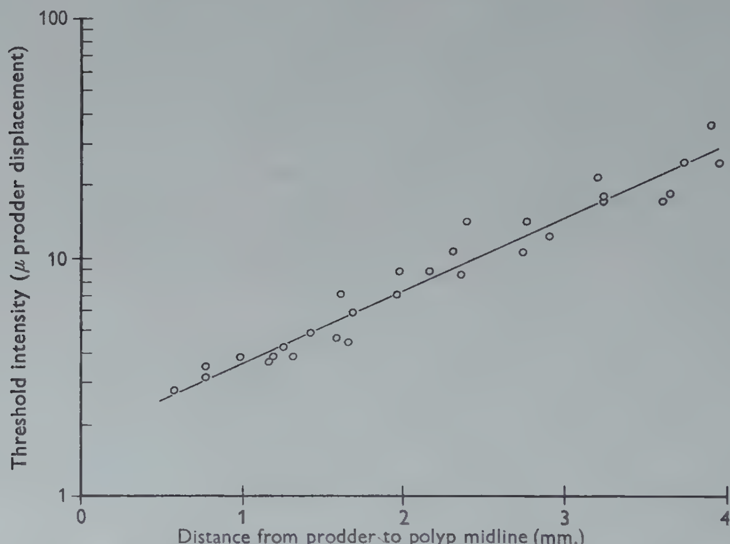

Fig. 2. Threshold intensity plotted against the distance from the prodder to the polyp mid-line. All data from one polyp, each point representing a single measurement.

These results show that pulses which cause no overt response can still modify the animal's future behaviour, somehow inhibiting the bending response to normally supra-threshold stimuli arriving in the immediate future.

(*e*) *Responses to water currents*

Because of the small distances involved, it is difficult to decide if *Syncoryne* responds to pressure waves, or to currents set up by the prodder movement. To distinguish between these possibilities, the reactions of *Syncoryne* to stimuli consisting mainly of water currents were observed.

A glass pipette was mounted in a manipulator, and a rubber bulb attached to the pipette by a flexible tube. The end of the pipette was then brought near to a polyp, and, by squeezing the bulb, the polyp could be subjected to water currents.

A polyp could be bent by the water jet until it was perpendicular to its stalk without responding. If the manipulator holding the pipette was tapped while the polyp was so displaced, it would often respond by bending toward the pipette.

By repeatedly squeezing and releasing the rubber bulb, a polyp could be made to oscillate about its stalk at frequencies up to three per second without responding by bending. If, however, the rubber bulb was compressed sharply, causing a sudden jet of water to strike the polyp (and probably also causing the system including the pipette to vibrate), the polyp usually bent toward the pipette.

These results indicate that *Syncoryne* polyps are quite inert to all but possibly suddenly changing water currents. Whether the polyps are sensitive to sudden changes in water currents streaming past them, or only to water-borne vibrations cannot yet be stated.

Mechanism of stimulus perception

For an animal to detect a mechanical disturbance, one portion of its structure must be moved by the stimulus with respect to some other portion. *Syncoryne*, unlike free-living animals, has a fixed position in space because of the attachment of its stolons to the substrate. It was thought possible that vibration perception in this hydroid involved displacement of the flexible hydranth about the more rigid, fixed, stalk. The following experiment was performed to test this hypothesis.

A viscous solution of methyl cellulose dissolved in sea water was placed in a dish. A layer of sea water was carefully poured over this solution and the two liquids slightly mixed, creating a liquid column of increasing viscosity and density. When a detached hydroid polyp was placed in this solution, it soon nearly stopped sinking. The polyp's vibration threshold could then be measured while it was thus suspended in a liquid and lacking a fixed point in space. Following such measurements, the cut end of the polyp stalk was grasped in a pair of forceps mounted in a manipulator, the polyp held at the same depth in the column, and the threshold again measured In the latter measurements, the polyps again had a fixed reference point. Table 1 gives the results of this experiment. Accuracy is defined as the number of times the polyp bent toward the plane of the prodder out of the total number of responses. If the polyp bent parallel to the prodder, away from the prodder, or contracted, the response was counted as negative.

Table 1. *Comparison of thresholds of polyps with and without a fixed point in space*

	Average threshold (μ of prodder displacement, prodder 1 mm. from polyp midline)	Standard deviation (μ)	Accuracy
Polyp floating in methyl cellulose (8 polyps, 15 measurements)	13·4	1·8	11/15
Polyp held by stalk in methyl cellulose (8 polyps, 20 measurements)	12·7	6·1	9/20

The results indicate that possession of a fixed reference point does not play a decisive role in *Syncoryne* vibration perception.

In order to determine the role, if any, of the capitate tentacles of *Syncoryne* in perception of a near-by disturbance, vibration thresholds of a number of polyps lacking tentacles were determined. Two polyps were pinned to a mounted piece of cork in each of several dishes. Both were anaesthetized with magnesium chloride and the tentacles of one were removed with a pair of fine scissors. After the polyps had recovered, an equal number of threshold determinations was made on each member of the pair, the intact polyp serving as a control. The results of this experiment are summarized in Table 2.

Table 2. *Comparison of thresholds of polyps with and without tentacles*

	Average threshold (μ of prodder displacement, prodder 1 mm. from polyp mid-line)	Standard deviation (μ)	Accuracy
Polyps with tentacles (14 polyps, 45 trials)	5·8	3·9	33/45
Polyps without tentacles (14 polyps, 45 trials)	10·7	7·9	19/45

The average threshold of polyps without tentacles was statistically significantly higher than those with tentacles ($P < 0{\cdot}001$).

In a second series of trials, all the tentacles were removed from one side of a number of polyps. A comparison of the thresholds when the prodder was on the side with tentacles and when it was on the side without tentacles was made. The results of this experiment are presented in Table 3.

Table 3. *Comparison of thresholds of half detentacled polyps when prodder is on the side of the polyp with tentacles and when it is on the side of the polyp lacking tentacles*

	Average threshold (μ of prodder displacement, prodder 1 mm. from polyp mid-line)	Standard deviation (μ)	Accuracy
Prodder on side with tentacles (14 polyps, 23 trials)	3·7	0·95	23/23
Prodder on side without tentacles (14 polyps, 23 trials)	4·6	1·65	4/23

The average threshold when the prodder was on the side with tentacles was significantly lower than when the prodder was on the side without tentacles ($P < 0{\cdot}05$). Most striking, however, was the difference in accuracy, 100 % in the first case, 17·4 % in the second. This compares with a normal accuracy in intact animals of about 80 %. The lower average threshold between the groups in the last study and the controls in the previous series might be due to different states of nutrition, different degrees of laboratory acclimatization, or possibly, a general heightened excitability due to the operation itself.

Further evidence of an increased tendency to bend toward the tentacle side was seen when a dish containing four such half denuded polyps was jarred. All four polyps immediately bent toward the side with tentacles.

In a further attempt to identify structures responsible for vibration perception, selected points on the polyp surface were subjected to discrete prods. The stimulating apparatus used was the same as described above, except that the prodder ending in a large flat surface was replaced by one which ended in a narrow blunt tip. This prodder was moved toward the desired portion of the hydroid until it touched and caused a small displacement of the hydranth. The threshold voltages of pulses activating the prodder system were then determined.

Three measurements were made at different times for each of the selected points on the polyp surface and tentacles, and these were averaged to get the threshold for this area. All measurements were made on a single polyp. The results of this experiment are presented in Table 4.

Table 4. *Comparison of the thresholds of tentacles and several points on the hydranth surface to discrete prods*

	Average threshold (μ of prodder displacement)	Standard deviation (μ)
Prodder on tentacles (average of 9 tentacles)	2·84	0·97
Prodder on hydranth surface (average of 6 points)	4·48	1·76

The average threshold of the tentacles was significantly lower than that of the points on the hydranth tested ($P < 0{\cdot}05$).

The direction of polyp bending was found to depend not on the direction of tentacle displacement, but only on the particular tentacle stimulated. The polyp bent toward the stimulated tentacle whether it was displaced toward the polyp body or in any direction perpendicular to the tentacle axis.

That such polyp responses are not only interesting laboratory phenomena, but may also play an important role in the life of the animal, was shown by experiments in which a small living copepod was held by the abdomen in a pair of forceps attached to a manipulator and brought near but not touching a *Syncoryne* polyp. As long as the copepod remained still, the *Syncoryne* was also motionless; as soon as the copepod began to struggle it was quickly seized and eaten by the polyp. A *Syncoryne* polyp can, then, detect and suitably respond to the presence of a small moving crustacean in its immediate environment. Even after having eaten a copepod (and being distended from the presence of such a relatively large object in its gastric cavity), a polyp would continue to respond to a vibrating rod.

DISCUSSION

With the information at hand, it is possible to set up a hypothetical model which would explain the bending behaviour in *Syncoryne*. This model can serve as a first approximation, and will certainly be subject to later revision.

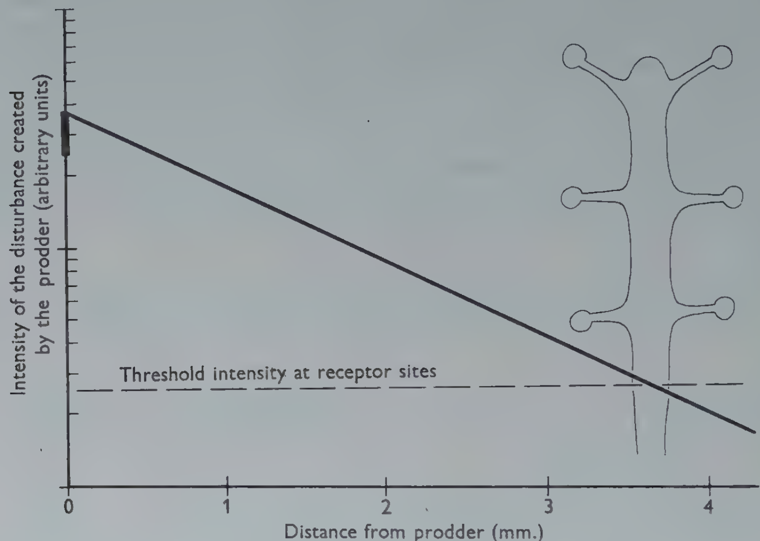

Fig. 3. The decrease in the intensity of the disturbance created by the prodder with increasing distance from the prodder. The linear log intensity *vs.* distance relation can be derived from the threshold intensity *vs.* distance curve (Fig. 2), if the assumption is made that the percentage decrease in intensity is a function only of the distance from the source. With the intensity at zero distance as given in the figure, and a polyp at the distance shown, the intensity will be above threshold on the side of the polyp toward the prodder (left), while still below threshold on the side away from the prodder (right). The vertical bar on the ordinate indicates the initial intensity range over which this condition would exist.

It has been shown that the intensity of the disturbance caused by the prodder falls off with increasing distance (Fig. 2). If the prodder is set at a given distance and the intensity of the stimulus gradually increased, when the disturbance is just above threshold on the side of the polyp closest to the prodder it may still be below threshold on the far side. It is postulated that the longitudinal muscles of the polyp are functionally divided into a number of parallel fields, each responding to disturbance only of its own periphery. In such a case, when the disturbance is above threshold on only one side of the hydroid, the polyp will bend toward that side (Fig. 3). If the polyp response is not all-or-none, but graded with the stimulus intensity (evidence of this is given below), the polyp will bend towards the prodder as long as the stimulus strength and degree of muscular contraction are greater on the prodder side than on the far side of the polyp, even if the stimulus intensity is above threshold on both sides.

The stiff polyp tentacles have been shown to be either directly sensitive to displacement or able to transmit a disturbance to sensory elements elsewhere on the polyp body. Since they project out from the polyp in all directions, one or more of them will be closer to the source of disturbance and hence subject to greater displacement than any other portion of the polyp. This, coupled with the greater sensitivity of tentacles than other areas on the polyp surface to displacement, makes it seem probable that it is movement of the tentacles which normally initiates the bending response. The lateral projection of the tentacles would also tend to increase the range of stimulus intensity over which only one side of the polyp is activated (see Fig. 3).

For such a mechanism to be a reasonably accurate control of the direction of bending, the stimulus intensity difference across the polyp must be significant when compared to the natural variation in sensitivity of either side of the polyp. The stimulus intensity at that part of the polyp closest to the prodder can be expected to be about 33 % higher than at the polyp midline (from Fig. 3). In the experiment shown in Fig. 2, 73 % of the threshold measurements fell between 16 % below and 16 % above the least squares regression line, showing that, indeed, the intensity difference across a polyp is significant when compared to the variability in sensitivity.

This model would explain the increased threshold when all the tentacles are removed from a polyp or when the prodder is on the tentacle side of a polyp from which half the tentacles have been removed. It also accounts for the decreased accuracy in polyps without tentacles (the width is decreased, hence there is smaller intensity difference on the two sides of the polyp and greater probability of exciting the far side or both sides simultaneously with near-threshold stimuli). A lower threshold for tentacle displacement than for displacement of other polyp parts would explain the tendency of half tentacled-polyps to bend towards the tentacle side and the great difference in the accuracy of such polyps with different prodder positions.

Further evidence that it is stimulus intensity difference on the two sides of the polyp which governs the direction of bending is given by experiments in which the stimulus intensity was increased above threshold with the prodder at a set distance from the polyp mid-line. Although there was too much variation in polyp response to give quantitative results, in general the degree of polyp bending increased with increasing stimulus intensity until about twice threshold strength. Above this, the angle to which the polyps bent decreased with increasing stimulus intensity. This decreased bending was associated with polyp shortening. At very high intensities the only response which could be produced was polyp contraction.

The interpretation given these results is that the degree of muscular contraction is a function of the stimulus strength. Increasing the stimulus strength leads to a greater contraction of the muscles on the side of the polyp towards the prodder. With further intensity increase, however, the displacement threshold for receptors activating the muscles on the far side of the polyp is surpassed. The degree of bending to still stronger stimuli will depend on the difference in the degree of contraction of the two sides of the polyp, until, with maximum stimuli and maximum

muscle contraction, the only response is a shortening of the hydroid. When both sides of the polyp are stimulated equally, as when the prodder is directly above the polyp, the response seen is equal contraction of all longitudinal muscles and polyp shortening.

The bending response of *Syncoryne* suggests an advantage gained from possession of short, stiff, tentacles and, hence, their *raison d'être* from an evolutionary point of view. The long slender tentacles of most hydroids would tend to drag behind a bending hydranth and be of little value in prey capture of this sort. The tentacles of *Syncoryne*, on the other hand, can be moved through the water rapidly and make effective weapons.

SUMMARY

1. *Syncoryne mirabilis* polyps rapidly bend toward the source of a mechanical disturbance in their vicinity. A small moving copepod a few millimeters from the polyp creates sufficient stimulus to evoke this response.

2. The hydroid will respond to a mechanical pulse from an electrically driven prodder as well as to single movements of the prodder either toward or away from the polyp. With the prodder 0·5 mm. from the polyp mid-line, threshold displacement of the prodder is only 2·5–3 μ. Repeated pulses of increasing amplitude, initially below threshold intensity, will not evoke bending if the frequency is over 5/sec. This means that stimuli which cause no overt response can still inhibit polyp bending to normally supra-threshold subsequent stimuli.

3. A polyp will not respond to slow currents of water, but will bend towards the source of sudden jets of water directed at it.

4. The capitate tentacles are not necessary for the movement perception, but after their removal the threshold is raised and the accuracy of bending reduced. The response does not require that the polyp be attached to a fixed stalk; it also occurs in isolated polyps floating in a density gradient.

5. The hypothesis is presented that polyp bending is due to unequal stimulation of opposite sides of the polyp because of decreasing stimulus intensity with increasing distance from the source of disturbance.

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EFFECT OF ACCLIMATIZATION TO HIGH TEMPERATURE ON THE BLOOD CHLORIDE, FREE AMINO ACIDS AND OSMOTIC PRESSURE IN THE FRESHWATER FIELD CRAB *PARATELPHUSA* SP. AND THE FRESHWATER MUSSEL *LAMELLIDENS MARGINALIS*

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INTRODUCTION

It is well established that the metabolism of organisms is size-dependent (Zeuthen, 1955), and that in poikilotherms it is greatly influenced by temperature (Krogh, 1916; Pampapathi-Rao & Bullock, 1954). Further, there is considerable evidence (Bullock 1955) which shows that several poikilotherms can acclimatize to temperature change by suitable compensation in their metabolism. The organ or cellular basis of this homoeostatic mechanism is not well understood, although there is some evidence for compensation at the cellular level and in the activity of enzymes (see Bullock, 1955, for a review).

In homoiosmotic organisms the composition of the blood (or the body fluid as the case may be) is important and is kept relatively constant. It is conceivable that the composition of the internal medium would be an important factor influenced by temperature changes in the environment since temperature would influence the osmotic processes of the organism. There have been few studies on the effect of temperature on osmoregulation and blood composition in invertebrates. One of the earliest studies which show such relationships is that of Loeb & Wasteneys (1912) who showed that *Fundulus heteroclitus* were better able to withstand osmotic stress at a high temperature if they were acclimatized for some time to that temperature. The studies of Wikgren (1953) are the major contributions in this field. There are some reports which indicate an influence of temperature acclimatization on the blood and blood constituents. For example, the number of red and white corpuscles in some fish is known to change in a regular manner with seasonal change in temperature (Schlicher, 1926; Spoor, 1951). While such studies as the ones cited are not size-controlled and systematic, they indicate sufficiently well that temperature acclimatization involves some changes in the internal medium. The present investigation shows that the osmotic pressure, chloride and free amino acid content of the blood undergo systematic change (under size-controlled conditions) during acclimatization to high temperature in the freshwater field crab, *Paratelphusa* sp. and the freshwater mussel, *Lamellidens marginalis*.

MATERIALS AND METHODS

Crabs were collected from the paddy fields, south of Tirupati, and were brought to the laboratory as soon as possible, so as to minimize the mortality due to overcrowding. Gravid females and injured animals were discarded. The others were placed in troughs containing water just sufficient to immerse them. The crabs were left in the troughs for 2 or 3 days at the laboratory temperature so that they might become adjusted to the laboratory conditions. In the initial stages the rate of mortality was greater due to the change in the environmental conditions, but gradually the rate of mortality diminished. Once they were adjusted to the laboratory conditions they survived well for considerable lengths of time.

After 2 or 3 days they were transferred in lots of 12 from the laboratory temperature ($26 \pm 1^\circ$ C.), to troughs which were gradually heated to the experimental temperature ($33 \pm 1^\circ$ C.).

The crabs were kept at the constant temperature of $33 \pm 1^\circ$ C. for 10 days in order to acclimatize them to that temperature. The water in the trough was changed daily, being replaced with water at the same temperature. At this higher temperature the rate of mortality in the initial stages was high, but gradually it decreased.

The animals were not fed one day prior to experimentation, and it was hoped that variations in their blood composition due to differences in feeding would thus be eliminated (Gilbert, 1959*a*, *b*, *c*).

After 10 days at $33 \pm 1^\circ$ C., the crabs were used for analysis. Since there is accumulation of calcium just prior to moulting (Robertson, 1937) and since the blood composition varies during the moulting cycle (Baumberger & Olmstead, 1928), measurements of osmotic pressure, etc. were only made on animals in the intermoult stage.

For each experiment crabs were selected so as to cover the maximum possible size range. The crabs were blotted to remove the water adhering to their bodies and were weighed on a Pelouze balance to the nearest 0·5 g. Blood was drawn from them by inserting a heparinized syringe through the arthrodial membrane at the base of one of the legs. The chloride, free amino acids and osmotic pressure of these samples of blood were determined.

Freshwater mussels were collected from a pond nearby, on the eastern side of Tirupati. After they were brought to the laboratory, they received the same treatment as described above for the crabs.

After 12 days at $33 \pm 1^\circ$ C. each mussel was weighed on a Pelouze balance. The wet weight of the soft parts alone was taken into consideration. Blood was drawn from the heart by inserting a syringe into the heart directly, avoiding contamination with the pericardial fluid as far as possible. These blood samples were used for the determination of the chloride and free amino acid contents.

Blood chloride was determined by Sendroy's method, as modified by Robertson & Webb (1939). One ml. of the sample taken was treated with silver iodate, the displaced iodine was dissolved in potassium iodide and titrated with sodium thiosulphate until two consecutive readings agreed.

Free amino acid content was determined on deproteinized blood by the Folin–Danielson method (Hawk, Oser & Summerson, 1954) using a Duboscq colorimeter. In this method the free amino acids present in the blood react with β-naphthoquinone-4-sulphonic acid, developing colour.

The osmotic pressure was determined by the well-known Barger's method (Barger, 1904, as modified by Krogh). The principle is that in an enclosed space solutions of higher concentration grow at the expense of solutions of low concentration.

RESULTS

The freshwater field crab, Paratelphusa *sp.*

The blood chloride values are plotted against the body weight on a double logarithmic graph, and these results were compared with the chloride content of the crabs at ordinary temperature, reported by Padmanabhanaidu & Ramamurthy (1961) (Fig. 1A).

In general, at ordinary temperatures, the females tend to have a higher chloride content than the males. The temperature has a profound effect on the chloride content of the blood. At high temperatures there is an over-all decrease in the chloride content in both the males and females, as compared with the chloride content at ordinary temperatures.

The amino acid values are plotted against the body weight on a double logarithmic graph. A comparison of these values with values obtained from crabs at room temperature shows that on acclimatization to higher temperatures these animals, irrespective of their sex and size, tend to retain a much smaller amino acid content in their blood than at ordinary temperatures (Fig. 1B).

The blood osmotic pressure is expressed as percentage sodium chloride solution. The results are plotted against the body weight on a double logarithmic graph. However, a comparison of these results with measurements made at ordinary temperatures indicates, *prima facie*, a general tendency for the lowering of the blood osmotic pressure in both the sexes (Fig. 1C).

Freshwater mussel, Lamellidens *marginalis*

Fig. 2 shows that the mussel, unlike the crab, shows considerable increase in the chloride and the free amino acids of the blood on acclimatization to high temperature. But the osmotic pressure increases only a little when compared with the values at normal temperatures.

DISCUSSION

It is seen from the results reported above that acclimatization to high temperature results in a change in the blood composition. The direction of change is not the same in the two species investigated. In the crab the three factors studied show a decrease in concentration, in both the sexes. But the decrease in the total osmotic pressure of the blood does not appear to be as great as would be expected from the combined effects of the decrease in the chloride and in free amino acid content of the blood.

On the other hand there is an increase in all the three factors studied in the freshwater mussel after acclimatization to high temperature. As may be seen from Fig. 2 the increase in free amino acid content of the blood is considerable over the whole size range investigated, while the increase in the chloride content is greater in the larger individuals. However, the total osmotic pressure of the blood does not show any great increase over the normal, and what little increase there is is mainly noticed in the smaller individuals.

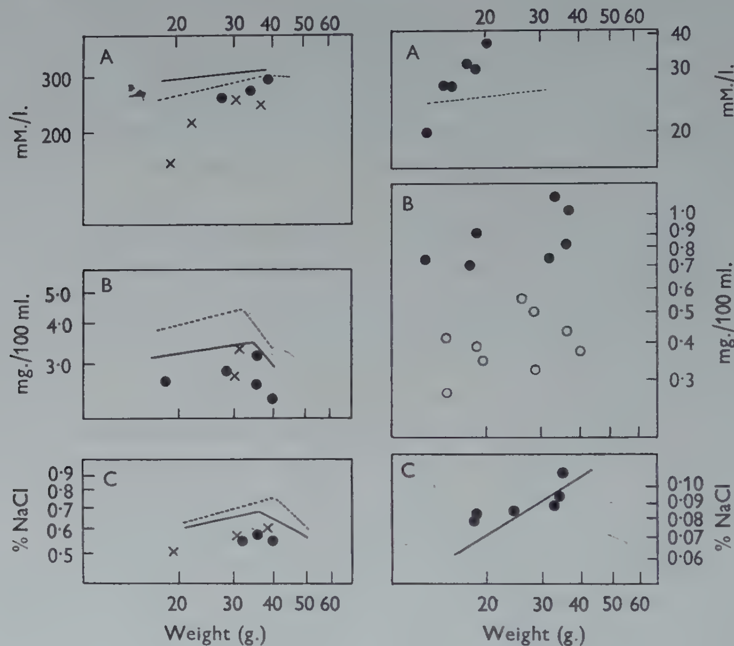

Fig. 1 Fig. 2

Fig. 1. Comparison of the blood constituents of the freshwater field crab *Paratelphusa* sp. at temperatures 26 ± 1° C. and 33 ± 1° C. In the figure, closed circles refer to the females and crosses to the males both at the higher temperature. The broken line refers to the males and the solid line to the females both at the lower temperature. Lines taken from Padmanabhanaidu & Ramamurthy (1961). A, Blood chloride; B, free amino acid content; C, osmotic pressure.

Fig. 2. Comparison of the blood constituents of the freshwater mussel *Lamellidens marginalis* at temperatures 26 ± 1° C and 33 ± 1° C. In A the broken line refers to the ordinary temperature, and the closed circles to the higher temperature. In B closed circles refer to the higher temperature and the open circles to the lower temperature. In C, the solid line refers to the lower temperature, and the closed circles to the higher temperature. Values for the lower temperature taken from Padmanabhanaidu & Ramamurthy (1961). A, Blood chloride; B, free amino acid content; C, osmotic pressure.

It is therefore seen from the above that the changes in the total osmotic pressure with acclimatization to high temperature are not attributable solely to the changes in the chloride and free amino acid content of the blood, irrespective whether these changes involve an increase (as in the mussel) or a decrease (as in the crab). Some other constituents of the blood also appear to be involved in these changes.

Systemic changes in relation to temperature acclimatization have not, hitherto, been investigated in any detail. It is, however, known that in lower vertebrates such as fish even the volume of the red blood cell changes (Kaplan & Crouse, 1956) as well as the number of cells (Schlicher, 1926; Spoor, 1951). Some data also (Cordier & Worbe, 1954) indicate slight increase in permeability in minnows acclimatized to low temperatures. Straub (1957) found a slight shift in the blood dissociation curve of the frog, which might be due to a change in the alkali reserve of the blood. The results presented above from two invertebrates indicate that the systemic changes noticed in the lower vertebrates may be related more basically to changes in the blood composition. While the direction of shift in the blood composition is not the same in the two cases studied above the mechanisms underlying these changes may be the same. The most well-developed hypothesis to account for temperature compensation in the activity of poikilotherms is that of Precht, Christophersen & Hensel (1955). This involves the relation between free and bound water. Increased resistance to thermal stress is readily explained by the reduction of free water. Increased thermal resistance in the active stages of insects is found to be associated with an increased concentration of the haemolymph. The changes in the blood concentration reported here for the mussel and the crab may also be related, although indirectly, to the amount of free-water available in the cells. Since these changes are in the haemolymph, it is apparent that the environment of the cell is also altered as a result of this acclimatization. Therefore, not only the osmotic and ionic relations between the blood and the medium, but also those between the *milieu intérieur* and the cells are altered. This gradient between the cells and the fluid surrounding them is important in the metabolism of the cells. It is therefore possible that the mechanism of metabolic compensation to temperature involves not only changes in the protein/water relations within the cell itself, but also the ionic and osmotic gradient between the *milieu intérieur* and the protoplasm of the cells.

SUMMARY

Acclimatization of the freshwater field crab, *Paratelphusa*, to high temperature results in a decrease in the chloride, free amino acids and osmotic pressure of the blood.

Following similar acclimatization the freshwater mussel, *Lamellidens marginalis* unlike the crab, shows a considerable increase in the blood chloride as well as the free amino acids, while the total osmotic pressure increases relatively little.

These results are discussed and it is suggested that the ionic and osmotic gradient between the *milieu intérieur* and the protoplasm of the cells might be important in the metabolic compensation to temperature.

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THE INFLUENCE OF SEX AND SIZE ON THE OSMOTIC PRESSURE, THE CHLORIDE AND THE FREE AMINO ACIDS OF THE BLOOD OF THE FRESHWATER FIELD CRAB, *PARATELPHUSA* SP. AND THE FRESHWATER MUSSEL, *LAMELLIDENS MARGINALIS*

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INTRODUCTION

An extensive literature is available on the ionic composition of the blood and osmotic regulation of marine as well as freshwater crustaceans (reviewed by Krogh, 1939; Panikkar, 1941; Prosser *et al.* 1950; Parry, 1957). But in most of the previous works, except for the very recent valuable contribution of Gilbert (1959*a–c*), the influence of sex and size on the blood composition has been overlooked. Gilbert (1959) has shown in *Carcinus moenas* that the blood conductivity and blood chloride increase steadily to a maximum at a weight of approximately 35 g and then decrease with the increase of body weight in both sexes, and that the females have higher blood chloride throughout the size range. But he has also shown that there is no difference in conductivity between males and females below 35 g., and that the blood conductivity tends to be higher in males than that in females above 35 g. body weight. He reports that the total osmotic pressure of male crabs is higher than that of females at any given body weight. But in both sexes the total osmotic pressure decreases steadily with increase in body weight. Gilbert showed that the curve (regression line) for total osmotic pressure has no peak at the middle of the size range, and he suggested that the discrepancy between ionic composition and the total osmotic pressure must therefore be due to non-electrolytes.

It is seen, therefore, that the only detailed study of this nature is confined to a marine crab. It would be of considerable interest to compare this with the conditions to be found in freshwater animals. The main purpose of the present investigation is to extend such a study of the osmotic pressure, blood chloride and free amino acids in the blood to such typical freshwater organisms as the freshwater field crab, *Paratelphusa* sp. and the freshwater mussel, *Lamellidens marginalis*. Since the freshwater mussel is a hermaphrodite the osmotic pressure, blood chloride and free amino acids in the blood of this mollusc have been studied in relation to its body size only.

MATERIALS AND METHODS

The crabs were collected in the paddy fields which are located to the southern side of Sri Venkateswara University campus. They were brought to the laboratory as soon as they were collected. The gravid females and injured animals were discarded. The remaining crabs were placed in groups of twelve each in glass troughs containing a small volume of water. The volume of water was adjusted such that the crabs were submerged under water. The initial mortality was relatively great but mortality was low after a few days, by which time the crabs had become adjusted to the laboratory conditions. The dead ones, if there were any, were removed and the water was renewed daily. Once they were adjusted to the laboratory conditions the crabs lived for long periods of time, provided they were fed. They were not fed for one day before the actual commencement of the experiment. This was to eliminate the variation in the blood composition due to differential feeding.

The composition of blood varies during the moult cycle (Baumberger & Olmstead, 1928) and there is a considerable increase in the calcium content of the blood (Robertson, 1939) just before moulting. For this reason measurements of osmotic pressure, blood chloride and free amino acids in the blood were confined to animals in the intermoult stage. The animals just after moult were also discarded; these were recognized by the softness and pale colour of the carapace.

The crabs were removed from the glass troughs and the excess of water was drained off, since it adds to their weight. Each was weighed in a Pelouze metric balance to the nearest 0·5 g. The sex and body weight of the crab were noted.

The blood was drawn through a small incision in the arthrodial membrane at the base of the fourth thoracic appendage. Sufficient blood was obtained by means of a hypodermic syringe for the measurement of osmotic pressure, blood chloride or free amino acids in the blood. For the determination of osmotic pressure an anti-coagulant, heparin, was used but not for the estimation of blood chloride and free aminoacids.

For the measurement of osmotic pressure, the classical Barger's capillary vapour pressure method modified by Krogh was employed (as described by Indira, 1960). The principle is that the osmotically stronger solution grows at the expense of the weaker solution. If the solutions are equally strong there is no increase of the length of either of the droplets. The osmotic pressure of the blood is expressed in terms of percentage sodium chloride.

Blood chloride was determined with 1 ml. of the sample and the method employed was that of Sendroy modified by Robertson & Webb (1939). The silver iodate was prepared in the laboratory according to the procedure suggested by Robertson & Webb (1939).

To determine the free amino acid content of the blood, the method of Folin and Danielson (Hawk, Oser & Summerson, 1954) was followed.

The freshwater mussels were collected from a small enclosed pond called 'Mangala Gunta' which is situated on the way from Tirupati to Alwar Theertham (Kapila Theertham). They were brought to the laboratory and placed in groups of

twelve in glass troughs containing water. While collecting, care was taken to collect the widest size range of the available animals.

Before the experiment was begun the animal was weighed entire. One of the shells was removed and the water drained off. Blood was drawn from the heart by means of a hypodermic syringe. Heparin was used for the determination of the osmotic pressure but not for the determination of blood chloride and free amino acids. The weight of the shell was substracted from the total weight so as to get the weight of the soft parts only.

RESULTS

Blood osmotic pressure. The osmotic pressure was expressed in terms of percentage sodium chloride solution. The values were plotted against the body weight for thirteen males in Fig. 1 A and for twelve females in Fig. 1 B. From the data it is clear that the percentage sodium chloride value reaches a maximum at about 0·825% for male crabs of 37 g. weight and 0·75% for female crabs of 31 g. weight.

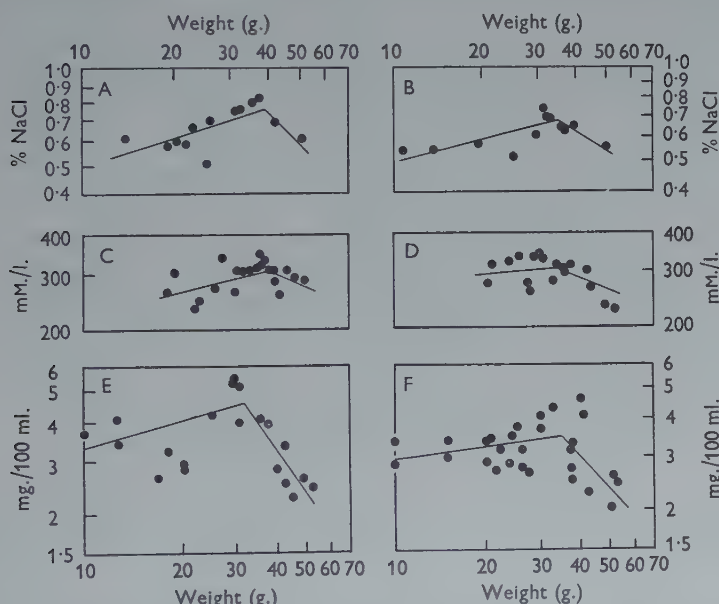

Fig. 1. Osmotic pressure, chloride content and free amino acids of the blood of *Paratelphusa* sp. as a function of sex and body weight. A, Blood osmotic pressure in males; B, blood osmotic pressure in females; C, blood chloride in males; D, blood chloride in females; E, free amino acids in males; F, free amino acids in females.

For statistical analysis the data for each sex were divided into two groups. In the case of males, the animals whose weight was below 37 g. formed one group and those above 37 g. a second group. Similarly, the females whose weights were below 31 g. formed one group and those above 31 g. a second group. Statistically, the lines of best fit were obtained by calculating the regression coefficients by the principle of least squares. In the case of male crabs the osmotic pressure rises

steadily with increase in body weight up to 39 g. weight and then falls significantly with further increase in size. But in the case of females the osmotic pressure rises gradually up to 35 g. weight. In general, the males tend to have a higher osmotic pressure than females over the whole size range.

A close examination of the Fig. 1 A and 1 B reveals that the difference in the osmotic pressure between sexes was not significant at their minimum body weights. But this difference is quite significant at the weights corresponding to their maximum osmotic pressure. This difference is still present even in the negative slope of the regression lines; but it is not as great as it is seen at 39 g. and 35 g. weight of males and females respectively.

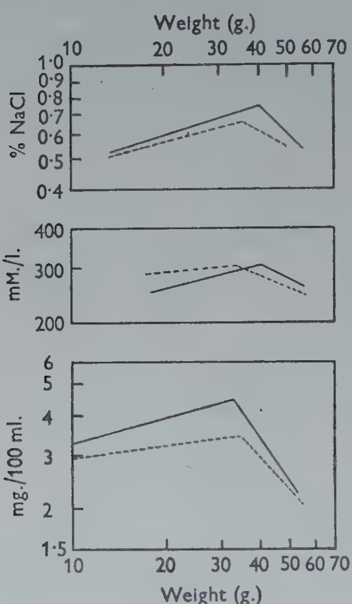

Fig. 2

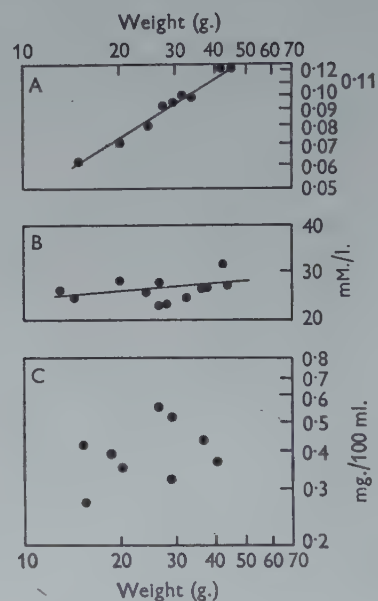

Fig. 3

Fig. 2. A comparison of the males and females of *Paratelphusa* sp. in respect of osmotic pressure, chloride and free amino acid content of the blood. Curves taken from Fig. 1. Solid lines for males. Broken lines for females.

Fig. 3. Osmotic pressure, chloride content and free amino acids of the blood of *Lamellidens marginalis* as a function of body weight. A, Blood osmotic pressure; B, blood chloride, C, free amino acids of the blood.

The values obtained for the blood osmotic pressure of the freshwater mussels are plotted against the body weight for nine animals in Fig. 3A. From the data it is clear that there is a significant increase in the osmotic pressure with increase in size of the animals.

Blood chloride. The blood chloride values in mM./l. were plotted against the body weight for twenty one males in Fig. 1C and for eighteen females in Fig. 1D. The trend of variation in the blood chloride with body weight and sex seemed to be

similar to that of osmotic pressure. From the data it is clear that the maximum blood chloride in males was at 38 g. weight and in females at 30 g. weight with a decrease on either side of these points.

As in the case of osmotic pressure the data for each sex have been split up into two groups for statistical analysis, viz. above and below 38 g. weight for males and above and below 30 g. weight for females. The lines of best fit were drawn by calculating the regression coefficients by the method of least squares.

From Fig. 1 C and 1 D it is found in both sexes that the chloride content of the blood increases with increase in weight to a maximum and then gradually decreases. From the calculated lines drawn it is seen that the maximum blood chloride is at about 40 g. in males and 35 g. in females.

A close examination of the Fig. 1 C and 1 D reveals the following facts. It is rather surprising to find that in the positive slope of the regression line the females tend to have a high blood chloride and in the negative slope of the regression line the males tend to have a high blood chloride. The difference in the blood chloride is significant in the smaller crabs, the females having a higher chloride content. In the negative slope of the regression lines the males above 40 g. have higher blood chloride, but this may not be significant. But if the negative slopes of the regression lines of both sexes are extrapolated towards higher weights the differences in blood chloride becomes insignificant, as was found with the blood conductivity in the lower weight ranges of both the sexes in the shore crab *Carcinus moenas* (Gilbert, 1959*a*).

The blood chloride of the freshwater mussel is plotted against the body weight for twelve specimens in Fig. 3 B. It is clear that there is a slight increase in the blood chloride with increase in size over the size range studied.

Free amino acids. The free amino acids present in the blood are expressed as mg./100 ml. of blood. The values are plotted against the body weight for twenty-two males in Fig. 1 E and for twenty-eight females in Fig. 1 F. The amino acid value reaches a maximum at about 32 g. weight in males and at 35 g. weight in females. As above, the data for each sex have been divided into two groups for statistical analysis, viz. above and below 32 g. for males and above and below 35 g. for females. The regression lines for the different groups are fitted as shown in Fig. 1 E and 1 F using the method of least squares.

In both sexes the free animo acids increase steadily with increase in size to a maximum and then decrease. In the male crabs the maximum free amino acid is found at about 32 g. and in females at about 35 g. body weight.

It is seen from Fig. 1 E and 1 F that in general the males tend to have a higher free amino acid content in the blood than females at any given weight. But the difference in free amino acids was conspicuous in individuals of 32 g. or less while in individuals above this weight the difference was not found to be significant.

The free amino acids in fresh water mussels are plotted against body weight on a double logarithmic graph for nine animals in Fig. 3 C. Though there is a considerable variation in the amino acid values it is clear from the figure that there is a slight increase as the body weight increases.

DISCUSSION

The blood composition of the field crab *Paratelphusa* sp. varies noticeably with body weight and sex, as was described by Gilbert (1959) in the marine shore crab, *Carcinus moenas*.

In the common shore crab (Gilbert, 1959) the total osmotic pressure in both sexes decreased steadily with increase in size and males tended to have a higher osmotic pressure. In the present work the osmotic pressure was found to increase steadily with increase in size up to a maximum and then gradually to decrease. The maximum osmotic pressure in males was at about 40 g. and in females at about 35 g. weight. The males had significantly higher osmotic pressure at any given weight.

The pattern of blood chloride was similar to that of the shore crab (Gilbert, 1959) except that the smaller females had higher chloride values, while above 35 g. the males had slightly higher chloride values.

Osmotic pressure and blood chloride values when viewed together are of considerable interest. The males have a higher blood chloride. Since the osmotic pressure depends upon the number of free particles present in the blood the above observation is rather puzzling. It is probable that the slightly higher osmotic pressure of the blood of males might be due to the high free amino acid present in the blood of males. In addition, the high blood-chloride in the males in the negative slope of the curve combined with the slightly higher amino acid content in the same range explains the high osmotic pressure difference between males and females larger than 35 g. But the difference in blood osmotic pressure between males and females below 35 g. is relatively reduced on account of the higher blood chloride in females, although in this range the amino acid content of the blood of males is significantly higher than in females. The high amino acids of males in the positive slope of the curve and also the high chloride of males in the negative slope together contribute to the high osmotic pressure in the males. It is seen from the above that the pattern of blood amino acids might play a significant role in keeping the osmotic pressure of males at a higher level than that of females.

It has been shown above that the amino acid content of the blood reaches a peak at a smaller size in males than in females. Whether these differences in amino acid content are related to the maturity of the individual in *Paratelphusa* sp. is not known, since no attempt has been made to study growth in relation to sexual maturity in this crab. But in view of the studies of Gilbert (1959), wherein it was suggested that the variation in non-protein nitrogen of the blood of *Carcinus moenas* might be related to the sexual maturity of the crabs, it is possible that this may be the case in *Paratelphusa* sp.

In the freshwater mussel, in contrast to the field crab, the blood osmotic pressure, chloride and free amino acids show no maximal value in the middle of the size range. On the other hand, they show a slight but continuing increase with increasing size. In addition, the blood osmotic pressure, chloride and free amino acids were comparatively low in the freshwater mussel.

SUMMARY

1. The influence of sex and body size on the osmotic pressure, chloride and free amino acids in the blood of the freshwater field crab, *Paratelphusa* sp., and the freshwater mussel, *Lamellidens marginalis*, were investigated.

2. In the crab the osmotic pressure increased in both the sexes to a maximum at about 40 g. in males and at 35 g. in females and then fell with further increase in weight. Throughout the whole size range the males tended to have a higher osmotic pressure.

3. Blood chloride in the crab also increased with weight in both the sexes to a maximum at about 40 g. in males and 35 g. in females and then steadily decreased as the weight increased. In the positive slope of the regression line the females have a higher blood chloride, but in the negative slope of the curve the males have higher blood chloride.

4. The free amino acid content of the blood reached a maximum at about 32 g. in males and 35 g. in females and then gradually decreased as the weight increased. Over the whole size range the males tended to have higher free amino acid content than the females.

5. In the freshwater mussel both chloride and free amino acid content of the blood showed a small but steady increase with increasing weight. This is also reflected in a significant increase in the blood osmotic pressure with increase in body weight.

We are greatly indebted to Prof. Kandula Pampapathi Rao, Head of the Department of Zoology, Sri Venkateswara University, Tirupati, A.P., for suggesting this problem, and for his kind consideration at all times.

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RATES OF ESTABLISHMENT OF REPRESENTATIONS IN THE MEMORY OF OCTOPUSES WITH AND WITHOUT VERTICAL LOBES

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INTRODUCTION

Numerous experiments have shown the capacity of *Octopus* to learn to attack one figure and avoid another, but there has been no thorough study of the rate of learning under various conditions, such as the spacing of trials and the method by which the reward is given to the animals. In the present experiments a systematic investigation was made of the setting up in the memory of representations that promote or prevent attack, in sets of animals under controlled conditions. It thus becomes possible to compare the rate at which the representations are established in normal animals and in those lacking the vertical lobes. 'Memory' is here used to denote the neural network within which sets of connexions are established when certain sets of attributes recur in combination with stimuli such as food or pain that evoke specific responses. Such sets of connexions are then referred to as representations ensuring the appropriate responses (Young, 1959, 1960*a*).

The experiments were designed to answer the following questions:

A. Are representations ensuring attack at a figure set up only if food is given at the place where the figure is shown, or can they be established by showing the figure in one place and feeding in another?

B. At what rate are such representations set up with trials (*a*) at long intervals, and (*b*) at short intervals?

C. What are the rates of their formation after removal of the vertical lobes?

D. How does removal of the vertical lobes influence the time for which the effects of a shock in preventing attack can be detected?

METHODS

Octopuses were isolated in rectangular tanks with opaque sides and lids. They were given bricks as a home at one end. The figures were opaque white plastic rectangles shown moving up and down on a transparent rod, inserted through an opening in the lid at the end of the tank away from the home. The times between inserting the rectangle and completion of the attack were taken with a stop-watch.

Operations for removal of the vertical lobe and assessment of the amount removed were as described by Boycott & Young (1955*a*).

EFFECT OF POSITION IN WHICH FOOD IS GIVEN

Eighteen octopuses were isolated and tested for 3 days twice daily by showing crabs but not allowing these to be eaten. Attacks were made at about 8/10 trials. No food was given on these days. The animals were then divided into three sets. All were shown a white vertical rectangle 1 m. from the home, at 10 min. intervals, ten times daily, at morning and evening sessions (Fig. 1). The animals of set A were given

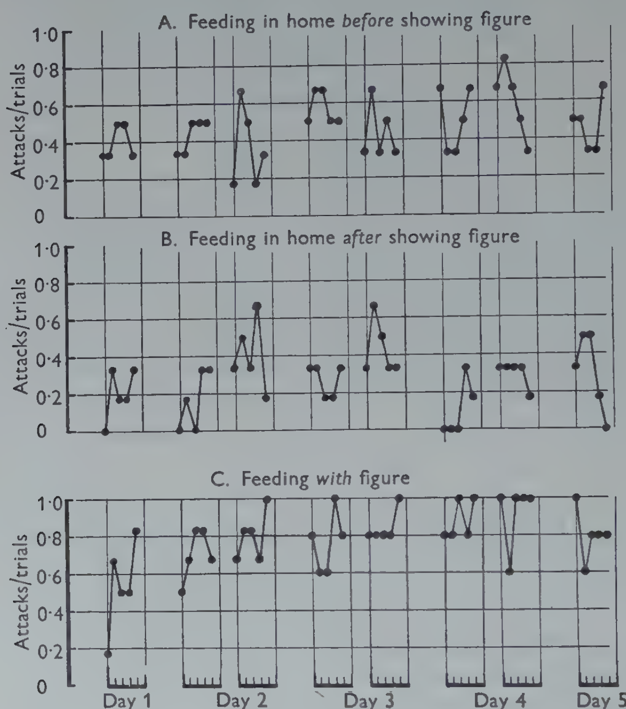

Fig. 1. Effect of place and time of reward on learning to attack a white vertical rectangle. Five normal octopuses in each group. Each point shows the proportion of animals that attacked when the rectangle appeared. See text. Only the animals in set C, where the reward was given close to the figure, learned to come out to attack.

a small piece of fish in the home immediately before showing the rectangle, the fish being introduced on the end of a wire. In set B the rectangle was shown until it was attacked, or for 20 sec., and food was then given to the octopus in its home, the rectangle being first pulled away if there had been an attack. The third set, C, were fed when there was an attack, or after 20 sec., the food being put near to the rectangle, so that the octopus seized both together. If there was no attack, even when the fish was put in, then fish and rectangle were moved towards the home until an attack occurred.

At the first trial of the first session the animals in groups B and C showed almost no attacks, no representations ensuring attack at this figure being present. In

group A there were some attacks, due to the stimulus of feeding (Young, 1958). At subsequent trials of the session there were more attacks in all three groups, but the effect was markedly greater in group C, where the food was given with the figure (Fig. 1). By the end of the first session of five trials the animals in this group were attacking 8/10 times. On this first day there was only one session.

The next morning there were no attacks by group B at the first trial, they had learned nothing from the day before. Group C, on the other hand, attacked at half the trials. In group A there were the same number of attacks as at the first trial of the first day. Group C was, therefore, the only one to show signs of an increased tendency to attack. Moreover, the mean time taken to attack began to fall steeply in these animals that were fed with the figure (Fig. 3).

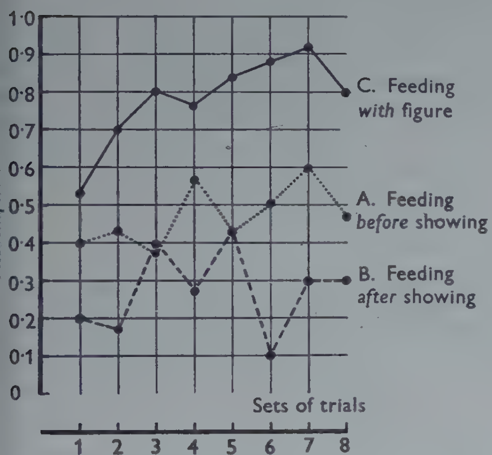

Fig. 2

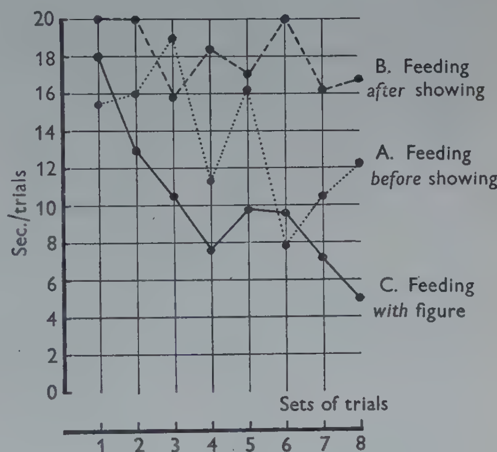

Fig. 3

Fig. 2. Mean proportion of attacks at each session of the experiment in Fig. 1.

Fig. 3. Mean delay before attack at the first trial of each session of Fig. 1. When there was no attack the time was taken as 20 sec.

This pattern of behaviour continued throughout the 5 days of the experiment. The animals in group C, where food was given with the rectangle, showed an increasing proportion of attacks at the first trial of each successive session, until finally attacks were made on the great majority of occasions (Figs. 1, 2). The mean time taken to make the first attack of the session fell from 18 sec. at the trial of the first session to 5 sec. at the eighth session (Fig. 3). The animals fed in the home after showing the figure (Group B) gave an irregular performance, with a slight tendency to increased numbers of attacks both at the first trial of each set and in the means for each set. There was no decrease in the times taken to attack. There was, therefore, at most a slight formation of representations promoting attack; after 5 days attacks still occurred only on 3/10 occasions.

The animals in group A showed rather more attacks than those in B, as is to be expected since the rectangle was always shown during the period of raised

excitability after feeding. However, the increase in proportion of attacks is about the same as in group B and far less than in group C. After 5 days, attacks were still only made on half the occasions. The times to attack at the first trials of each session were irregular and fell only from 15 to 12 sec.

This result was confirmed in another series of sixteen animals. For 5 days these were given food in the home, half of them before and half after showing a horizontal rectangle. At the beginning of the experiment both groups attacked only on 1–2/10 occasions. At the beginning of the fifth day those fed after showing attacked on 1/10, those fed before not at all. In neither group had representations ensuring attack been set up in the memory.

On the sixth day both groups were fed *with* the figure on three occasions. The number of attacks rapidly increased and on a fourth trial at the end of the day there were 9/10 attacks. The next morning there were attacks at about half the trials.

We may conclude that for the setting up of representations that promote attack at visible figures it is important that the food should be presented close to the figure, so that the two are seized together. Food given in the home before or just after the attack does not ensure the formation of such a representation in the memory.

SETTING UP OF REPRESENTATIONS PROMOTING ATTACKS, WITH TRIALS AT LONG AND SHORT INTERVALS

Twenty animals were isolated for 4 days and then operated, half for removal of the vertical lobe and half with a dummy operation. After allowing 2 days for recovery all were trained to attack a white vertical rectangle, half of each sort with trials at 5 min. intervals, the other half at 50 min. intervals. Food was given as soon as each animal attacked. If there was no attack within 15 sec. a piece of fish was placed near to the rectangle and the two were moved towards the octopus until the food was taken. There were eight trials each day, given at morning and evening sessions. Considering here the normal (dummy-operated) animals we see that there was an increase in frequency of attacks and decrease in their delay in both 5 min. and 50 min. groups (Fig. 4). The only consistent difference between the groups was that improvement in performance went on rather longer with the 5 min. trials, producing slightly higher criteria at the asymptote. There were consistent differences in behaviour between individuals, some learning more quickly to come out with a short delay. However, the pattern was the same for all. At the beginning none came out regularly and all showed long delays. By the end nearly all were very regular and the delays were much less. On some occasions all five animals in a set attacked with a delay of only 2 sec., which is the minimum that can be reliably recorded with this method.

EFFECTS OF VERTICAL LOBE REMOVAL ON SETTING UP OF REPRESENTATIONS PROMOTING ATTACKS

The animals without vertical lobes in the series in Fig. 4 showed no consistent increase in number of attacks or reduction in their time with training either at 5 min. or 50 min. intervals. This confirms previous evidence that representations ensuring

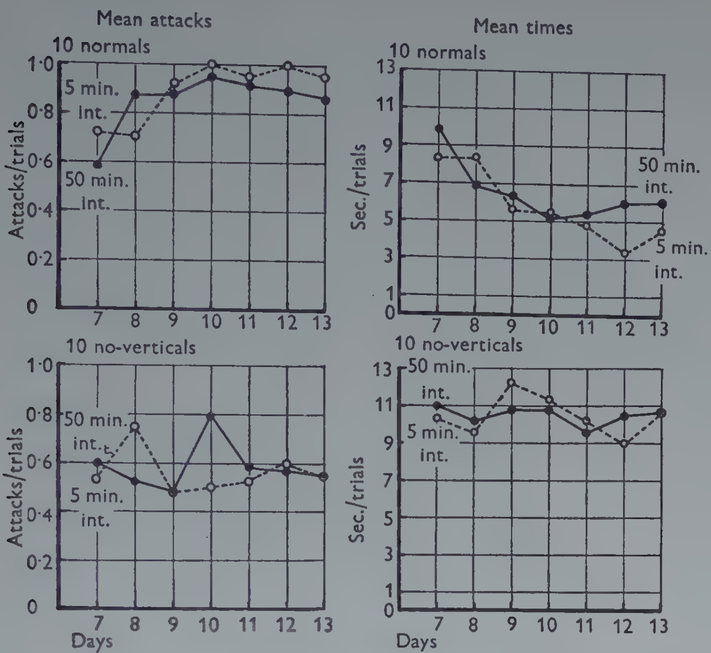

Fig. 4. Effect of interval between trials on learning to attack a white vertical rectangle. Open circles, trials at 5 min. intervals; filled circles, trials at 50 min. intervals. The points show the means for the ten trials each day. Above, ten normal animals; below ten with vertical lobes removed (94 %). Series KBA.

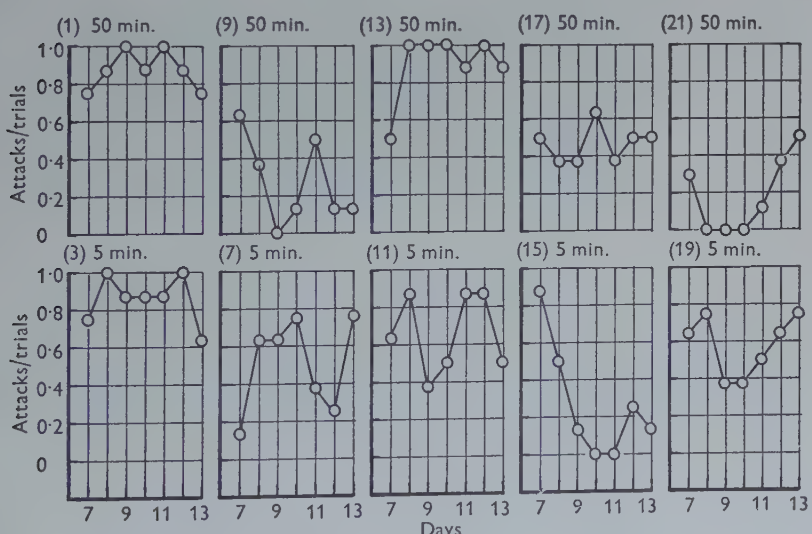

Fig. 5. Mean attacks per day by each of the individual operated animals of Fig. 4. The figures in brackets are the serial numbers of the animals.

attacks are set up in the memory only with difficulty after this operation (Young, 1960*a*). There were considerable differences between individuals in the tendency to attack (Fig. 5). Three attacked consistently throughout and the others fluctuated, all attacking sometimes, but none with steadily increasing frequency. There was no obvious pattern to the distribution of these attacks. An individual would show no attacks during the four morning trials and then come out at every trial in the afternoon. Then later its attacks would be quite scattered among the trials.

Injury to the lobes below the vertical lobe may affect the tendency to attack. None of these animals had serious lesions in these regions. The subvertical lobes were somewhat damaged in octopus 3, but this was one of the most persistent attackers. In all of them more than 90 % of the vertical lobe had been removed (mean 94 %).

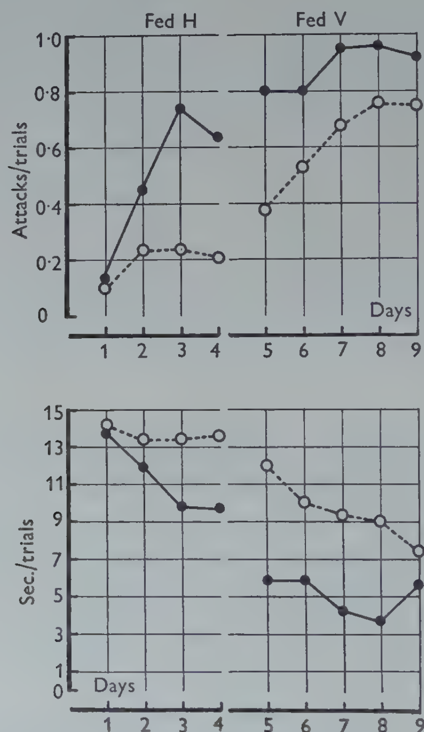

Fig. 6. Learning to attack horizontal and vertical rectangles by five normal animals (filled circles) and eleven without vertical lobes (open circles). 88 % had been removed. The points show the proportion of attacks made and the mean times during the five trials of each day, given at 15 min. intervals. Learning is much slower by the operated animals. Series JYA.

A similar result was seen in a further series of five normal and eleven no-vertical animals (Figs. 6, 7). On the day after operation (day 1) both groups tested with a white vertical or horizontal rectangle attacked either of these at about 1/10 occasions. For the next 3 days they were given five trials a day at which the horizontal rectangle was shown, and fish was given if attacks were made, or after 15 sec. if there was no

attack. The fish was given with the figure and the two were pushed towards the octopus if necessary. Trials were at approximately 15 min. intervals. The normal animals rapidly learned to attack the rectangle on about 7/10 occasions, though always with rather long delay. The animals without vertical lobes showed hardly any increase in proportion of attacks.

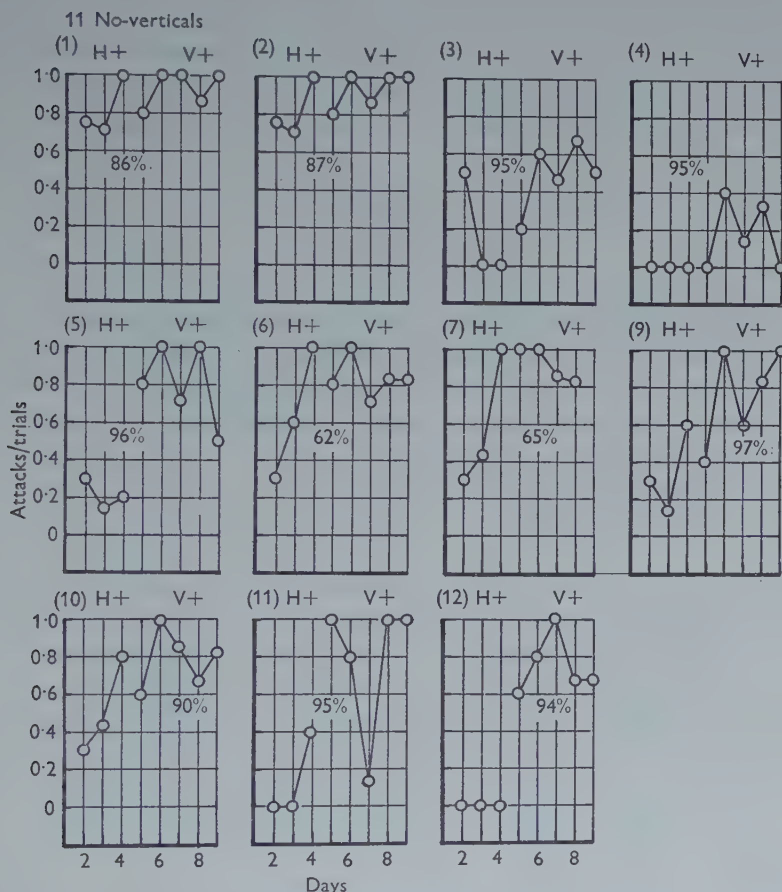

Fig. 7. Behaviour of the individual operated animals of the experiment of Fig. 6. The figures in brackets are the serial numbers of the animals.

Then from the 5th to 9th days similar tests were continued with a vertical rectangle. The normal animals attacked this about 8/10 times at first and soon came to do so almost always and with short delay. The no-verticals at first attacked about 4/10 times and then some of them gradually learned to do so, until the mean was nearly 8/10 times and the delay shorter (Figs. 6, 7). As before there were considerable and consistent differences between individuals. Two were persistent attackers throughout (numbers 1 and 2). Five became fairly persistent and two others attacked frequently but irregularly (numbers 5 and 11). Two attacked at less

than half the trials (numbers 3 and 4). There was no serious injury to the underlying lobes to connect with the tendency to attack. A mean of 88 % of the vertical lobes had been removed, with extremes of 62 and 97 %. There is no obvious correlation between the number of attacks and the amount of tissue remaining. The rate at which the representations were established in the memory was obviously much less than in the normal animals. No exact comparison of rates can be made, but after five occasions of feeding with the vertical rectangle the normal animals were attacking more reliably and with shorter delays than the no-verticals after twenty-five trials. By this criterion the memory system is at least five times less efficient after vertical lobe removal.

SETTING UP OF REPRESENTATIONS PREVENTING ATTACKS AT A RECTANGLE

The effect of shock in reducing the tendency to attack a previously 'positive' figure was tested in the series described in the last section by giving on the morning of each of the next 4 days three shocks following showings of the white vertical rectangle. On the first day every animal was shocked at each of the three trials, whether or not an attack had been made, the electrodes being introduced into the home if necessary. Thereafter no further shocks were given to the normal animals; the no-verticals were shocked only if they attacked.

In order to test the persistence of the effect of the shocks tests were then made 15, 30, 60 min, 2 and 4 hr. after the shocks on each day (Figs. 8, 9). As control, six of the no-vertical and two of the normal animals were tested throughout by showing the rectangle but not given any shocks. We thus distinguish between an 'extinction group' (the controls) and a 'shock group'. All the animals were fed with fishes each evening, some hours after the tests.

The normal animals, after they had been shocked three times, stopped attacking and made very few further attacks throughout the 4 days. The 'extinction group' of normals continued to attack in spite of the absence of rewards, though not at every trial. The animals without vertical lobes, after the first three shocks, stopped attacking but then mostly began again by the end of that day. Although the representations ensuring attack had been 'weaker' than those of the normal animals the tendency to attack was less affected by the shocks, because the process of setting up representations *preventing* attack was also weak. On the next (eleventh) day, however, fewer came out and on the 12th and 13th days attacks became still further depressed so that at the end none was attacking. Tests with crabs, however, then showed attack by nearly all the animals.

The control group of animals without vertical lobes, tested without shock, also showed some falling off in attack each day as a result of extinction, which has been shown to occur readily in octopuses without vertical lobes when the representation ensuring attack is 'weak' (Young, 1959).

However, these control animals recovered after extinction to a full level of 100 % attacks on each subsequent morning. The difference between the 'shock' and

'extinction' groups at the beginning of each day thus shows signs that there is some cumulative effect, even in these operated animals, of the shocks in setting up representations that prevent attacks. This process, was however, very much slower than in the normal animals, which after the shocks on the first day showed only rare attacks during the subsequent days. Exact comparison is difficult, but three shocks were sufficient to prevent attacks almost completely by the normals for 24 hr. and signs of the effect could be seen for much longer. In the no-verticals little or no effect of three shocks was seen 24 hr. later. Even after nine shocks many attacks were made. The process of forming representations in the memory that prevent attack is again at least five times less efficient without vertical lobes, using time of persistence as criterion.

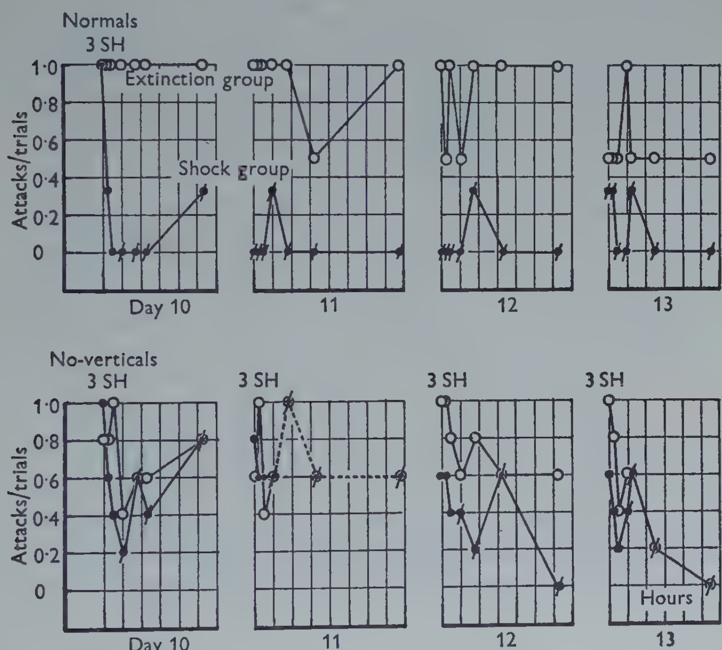

Fig. 8. Effect of shocks in preventing attacks at a 'positive' figure. The animals had all learned to attack a white vertical rectangle on days 1–9 as shown in Fig. 6. Then on day 10, three of the normals and five no-verticals were given shocks on three occasions after showing the rectangle (filled circles). Their responses were then tested by no-reward trials (crossed circles). The normal animals were given no further shocks but the operated ones received three further shocks at the beginning of each of the next 3 days. Two normal and five no-vertical animals provided control 'extinction' groups (open circles). These were shown the rectangle without reward or shock.

REPRESENTATIONS PREVENTING ATTACKS AT CRABS IN NORMAL ANIMALS AND AFTER VERTICAL LOBE REMOVAL

Normal octopuses that have received shocks following attacks at crabs soon cease to attack. The representation preventing attacks may then last for several days. In animals without vertical lobes it disappears more rapidly (Boycott & Young, 1955*b*).

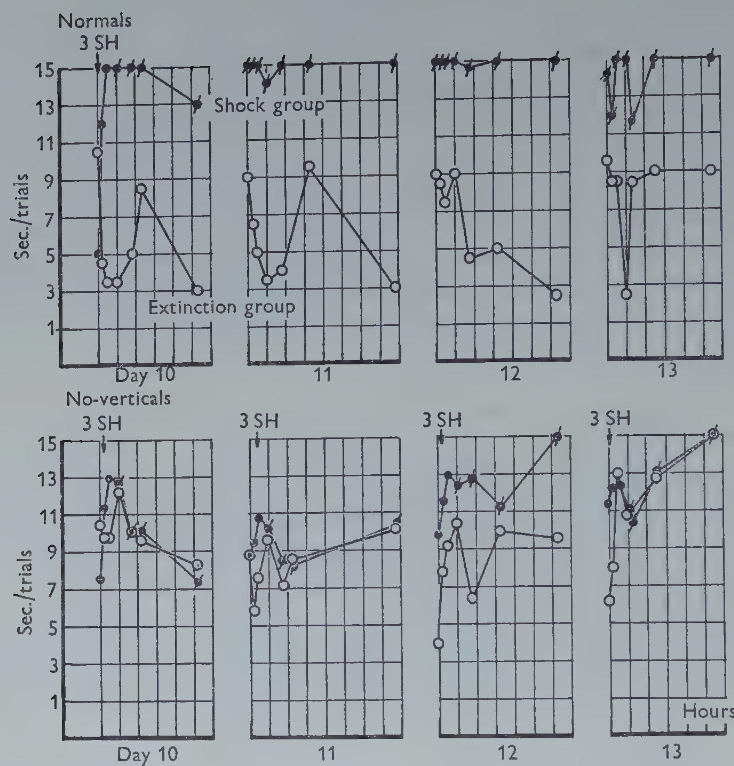

Fig. 9. Mean times to attack in the experiment of Fig. 8.

This has been confirmed in the present series by showing crabs to normal and no-vertical octopuses and giving shocks whenever there were attacks. The trials were given at 6 min. intervals, in sets of ten in the morning and ten in the evening of each day. As before, half the animals of series JYA were used as controls for the possibility of extinction, being simply tested with crabs, without reward or shock when there were attacks.

In the normal animals attacks ceased after each had received two or three shocks and were only resumed 3 days later (Figs. 10, 11). During the period when there were no attacks the white vertical rectangle was shown occasionally and was sometimes attacked. The representation preventing attack was thus at least partly specific to crabs. The normal animals that were given no shocks attacked on almost all occasions. The only signs of extinction were slight increases in delay (Fig. 11).

The animals without vertical lobes showed much greater persistence in continuing to attack crabs in spite of shocks. During each of the first ten trials at least one of the six octopuses in the 'shock' groups made an attack. No individual octopus attacked every time, each stopped and then after a few trials started again.

Seven hours later nearly all again attacked and the attacks fell off approximately as in the first set, that is, there was no clear sign of retention of the representations set up in the morning (Figs. 10, 11). However on the next day (day 15) the attacks

declined much more rapidly than before, and after the fifth trial no further attacks were made. Some information had therefore survived in the memories from the previous day. No such decline appeared in the five operated animals of the 'extinction' group, which were simply shown the crabs without reward and continued to attack on nearly every occasion, although not quite so regularly as the normal octopuses.

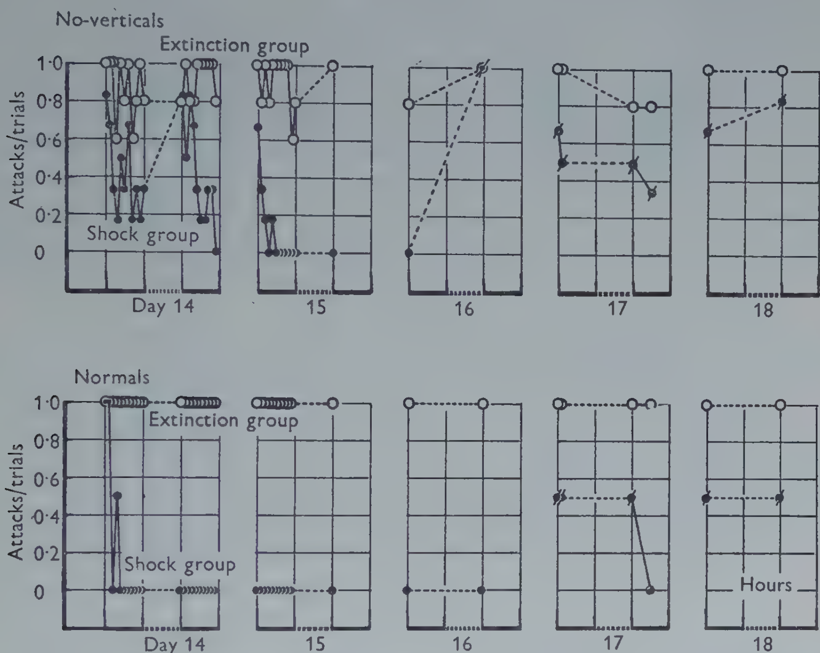

Fig. 10. Effect of shocks in preventing attacks at crabs. Continuation of experiment of Figs. 8 and 9 with division into groups to which shocks were given for every attack at crabs (filled circles) and extinction groups that were shown crabs but not allowed to eat them. After day 15 no further shocks. Trials on days 14 and 15 at 6 min intervals. On subsequent days tests in morning and evening (crossed circles).

No further shocks were given after day 15, but the animals were tested for 3 further days by showing crabs without reward, to discover the duration of the representations that prevent attack. The normal octopuses showed no attacks until the third day after the shocks and few even thereafter. The animals without vertical lobes, tested 7 and 24 hr. after shocks had last been given, showed no attacks. However, by 36 hr. many attacks were made by the shocked animals, though with longer delay than by the 'extinction' group, which had not been shocked (Fig. 9). Throughout the 3rd day after shocking, attacks were less frequent and slower by the shocked group and there were still some signs of a difference on the 4th day.

As explained on p. 50 a mean of 88 % of the vertical lobes had been removed from the whole group of eleven animals, without significant injury to the underlying lobes. The individuals that had been the most regular attackers during the period

of feeding with the vertical rectangle (numbers 1 and 2) were also among the animals that attacked crabs most persistently. However, even in these two the proportion of attacks became less by the 15th day.

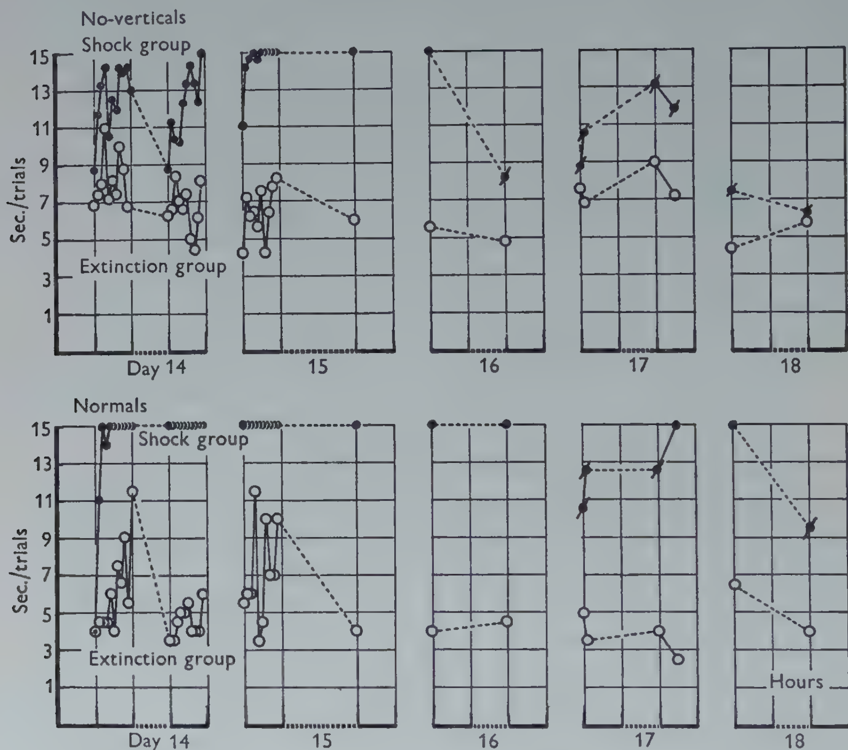

Fig. 11. Mean times to attack in the experiment of Fig. 10.

Representations preventing attack at crabs can thus be set up in the memory in the absence of the vertical lobes, but the process is much slower than in normal animals. More shocks are needed before attacks cease and, if the animals are left to forget, attacks begin again sooner in the operated animals. The no-vertical group required a mean of ten shocks before reaching a level at which no attacks were made for five trials. In the group of normal animals this level was realized after two shocks by one animal and three by the other. The memory is again at least five times more efficient in the normal animals.

Once established, the representations preventing attack remained fully effective in the operated animals for only 24 hr., though there were signs of its presence for 4 days. The data for the normal animals are inadequate, but there were clear signs of the effects of two shocks after 4 days.

EFFECT OF SHOCKS AT LONG AND SHORT INTERVALS IN PREVENTING ATTACKS IN ANIMALS WITHOUT VERTICAL LOBES

It has previously been suspected that the deficiency in learning by animals without vertical lobes lay in a failure to summate the effects of trials unless they were given close together (Boycott & Young, 1955*a*; Young, 1960*b*; Wells & Wells, 1957). Comparison was therefore made between the effects of shocks given at intervals of 6 and 60 min. The animals considered in the last section were used, some of which had already received shocks and others which had been used as controls on extinction with unrewarded tests. Two groups were now prepared; five in one and six in the other, both groups containing some that had and some that had not received shocks. Every animal was shown crabs four times and given a shock whether it attacked or not, in the latter case the shock being given in the home. For the six in group A the trials were spaced at 6 min. intervals, for the five in group B at 60 min. intervals. Thereafter all were tested by showing crabs without reward (Fig. 12).

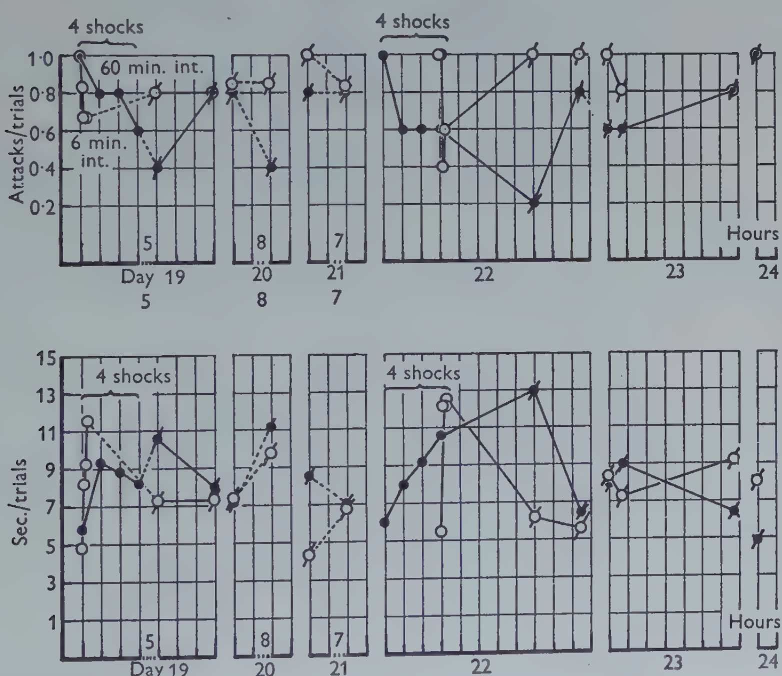

Fig. 12. Effect of shocks at 6 min. intervals (open circles) and 60 min. intervals (filled circles) in preventing attacks at crabs by animals without vertical lobes. The same octopuses as Fig. 10 but divided into two new groups. The shocks were given at the first four trials on days 19 and 22. At all the other trials crabs were shown but no reward or shock given when they were attacked (crossed circles). The effect of the shocks is more marked if they are widely spaced.

In group A there were altogether nineteen attacks out of the twenty-four occasions at which shocks were given (0·79), whereas in group B there were sixteen shocks out of twenty trials (0·80). During subsequent tests on the day of shocking

those given shocks at short intervals came out considerably more often and with shorter delays than those shocked at hourly intervals (Fig. 12). The same relationship was maintained during further tests without reward or shock on the following 2 days (20 and 21). By this time in both groups the level of attacks before shocking was nearly but not quite regained.

On the 22nd day, in order to repeat the experiment, the groups were reshuffled to form two new ones, each with some animals of the previous groups A and B. The new group C was given four shocks at 6 min. and D four shocks at 60 min. intervals. As before, the proportion of attacks during the shocking period was similar (0·75 for C, 0·70 for D), but the effect again lasted longer in those shocked at long intervals (Fig. 12). The difference had disappeared by the end of the next day (day 23).

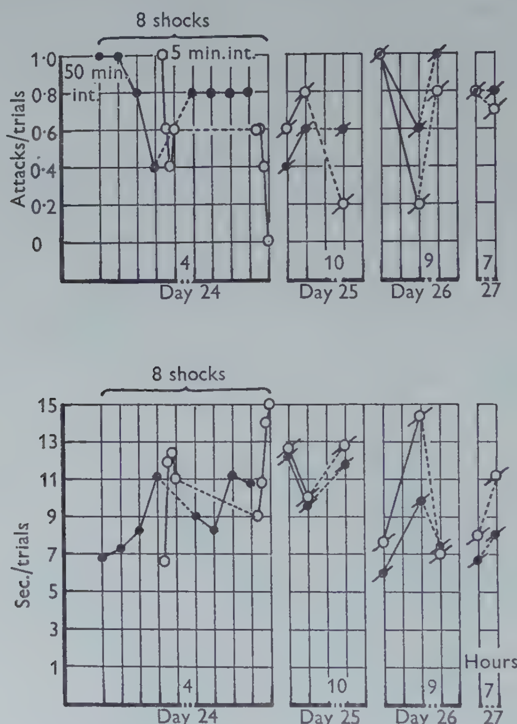

Fig. 13. Continuation from Fig. 12 with the animals re-arranged into two new groups. On day 24 eight shocks given at either 5 min. intervals (open circles) or 50 min. intervals (closed circles).

In order to repeat this experiment with a greater number of shocks the animals were again reshuffled on day 25 into groups E and F, each containing a similar number from C and D. Group E was given eight shocks at 5 min. intervals in two groups, four in the morning and four 7 hr. later. Group F was given eight shocks at 50 min. intervals, again in morning and evening groups (Fig. 13). This time the immediate depression produced by the shocks at short intervals was the greater. The animals shocked at short intervals attacked only at 0·525 of the trials as against

0·725 for the others. During the subsequent 3 days the frequency of the attacks and their delay were similar in the two groups, those receiving shocks at short intervals remaining somewhat less ready to attack.

From these experiments it is clear that there is no very marked difference between the effect of trials at long and short intervals in setting up representations that prevent attacks upon crabs. If anything a few shocks close together are less efficient than the same number separated by an hour in preventing attacks during the ensuing period. The results are summarized in Fig. 14, where the animals given shocks at long and short intervals are considered as two continuous series. By the reshuffling it was ensured that every animal had both treatments at some time during the experiment and the greater effect of the more widely spaced trials is therefore probably significant, for the condition that only a few shocks are given each day, but the differences are not great.

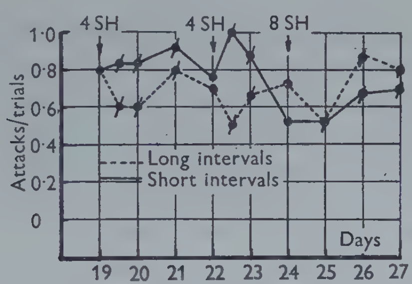

Fig. 14. Summary of the experiments of Figs. 10–13. Mean attacks on the occasions when shocks were given at different intervals and during the subsequent days. The shocks at longer intervals produced the greater effect when there were four of them. With eight there was little difference.

DISCUSSION

These experiments serve to establish some of the conditions under which representations promoting or preventing attack are set up in the memory of *Octopus*. The situation as regards learning to attack is complicated by the fact that these animals tend to come out to attack all objects more readily during the period after they have been fed. It has been established, however, that such attacks during the period of raised excitability do not result in learning to attack (Young, 1960*a*). This result is confirmed by the animals of Fig. 1A. Figs. 1B and C show the new point that feeding after showing is only effective if the food is given near to the figure. For survival it is reasonable that this should be so, there would be no point in learning to go out to attack if the food is to be found in the home. However, the mechanism by which this 'correct' result is secured remains obscure. It may be that there is a complicated interplay between the three sets of information provided by the object seen, touched and tasted. In the vertical lobe system impulses from these sources are brought together (see Young, 1960*b*), and this may be the means by which this lobe serves to promote the setting up of representations that ensure attack. However, as Figure 6 shows, such representations can be slowly formed in the absence of the vertical lobes. The optic lobes themselves receive tracts from the arms, both directly

in the brachio-optic tract and through the inferior frontal-optic tract (Boycott & Young, 1956).

The fact that learning takes place at much the same rate with rewards spaced at 5 and 50 min. agrees with experience with other animals. Pennington & Thompson (1958) found that rats learned a vertical-horizontal discrimination fastest with trials at 3 to 4 min. intervals, but between 5 min. and 24 hr. there was little further change. There is probably no general solution to this problem and it may be that for difficult problems distributed effort is more effective, for easy ones massed effort.

Learning not to attack an object that yields shocks involves bringing visual information together with that from pain receptors. This learning also can proceed slowly in the absence of the vertical lobes. The experiment of Fig. 12 suggests that shocks close together in time may actually be less efficient than more widely spread ones in these deficient animals. It has already been shown that part at least of the defect of these operated animals is that the effects of a shock in preventing attack wear off more rapidly than in normal animals (Young, 1960*a*). These two results are not necessarily inconsistent. For long-lasting learning to occur some trace must remain even when the animals have again begun to attack. In the summation of this with the traces left by subsequent trials it may well be that time itself is required for whatever process of growth establishes the connection. Further traces provided at intervals of say 1 hr. may thus be more effective than the same number provided at 5 min. intervals. Wells & Wells (1958) have discovered evidence that representations ensuring correct tactile response in *Octopus* only become fully established after a lapse of time.

The memory system appears to be at least five times less efficient after vertical lobe removal, as judged by the number of trials required to reach a given criterion of performance. The impairment appears to be of the same extent for the setting up of representations that ensure attack at a rectangle and those that prevent attack at a rectangle or a crab. In a previous study an impairment of 3–4 times was estimated by a somewhat different method (Young, 1960*b*).

In the daily life of the animal an impairment of this order would evidently have most serious consequences both for obtaining food and avoiding enemies. An octopus cannot afford to wait for a long process of learning such as humans use and psychologists often rely on for animal experiments. Octopuses have been shown to be able to make approximately correct discriminating reference to two figures after three or four trials (Young, 1960*a*). An increase by five times would be serious.

The capacity of the vertical lobes to produce this result is out of proportion to their size. They are estimated to contain 25 million cells (mostly very small), as against 60 million in each of the optic lobes. However, the latter are presumably primarily concerned with coding the information received from the retina, and they receive few fibres from the arms or other sources. In the superior frontal and vertical lobes all the relevant information is brought together. It is partly stored in them, but also passed back to the optic lobes in a form suitable for storage.

SUMMARY

1. The rate of learning to attack a rectangle seen at a distance was studied under various conditions.

2. Learning is rapid only if the food reward is given after the figure has been shown, and if it is near to it.

3. There was no learning to attack if the food was given in the home before showing the figure, nor if the food was given after the octopus had returned to its home.

4. There was no marked difference in rate of learning with trials at intervals of 5 and 50 min.

5. After removal of the vertical lobes learning to attack a rectangle occurred only slowly. Operated animals attacked less reliably after twenty-five trials than normal animals after five trials.

6. After being shocked three times following showings of a rectangle normal animals that had previously learned to attack made few further attacks during the next 4 days. Animals without vertical lobes began to attack again during the same day; little sign of the effects of shocks was seen after 24 hr.

7. The process of forming representations that prevent attack is estimated to be at least five times less efficient in animals without vertical lobes.

8. This was confirmed by the effect of shocks in preventing attacks at crabs. In normal octopuses attacks ceased after two or three shocks at 10 min. intervals and were only resumed 3 days later. Animals without vertical lobes attacked up to ten times in spite of shocks. A few hours later all attacked again. However, some evidence of more enduring representations in the memories was seen.

9. There was no great difference in the effect of shocks given at intervals of 5 and 50 min. in preventing subsequent attacks at crabs by animals without vertical lobes. Under some conditions the more widely spaced shocks produced the greater effect.

It is a pleasure to thank the Nuffield Foundation for the support that made this work possible, also Dr P. Dohrn and the staff of the Stazione Zoologica for all their help. Mr W. Muntz kindly assisted with the experiments on p. 44. Miss J. Parriss gave valuable help in the preparation of the paper.

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THE ACTION AND COMPOSITION OF THE SALIVA OF AN ASSASSIN BUG *PLATYMERIS RHADAMANTHUS* GAERST. (HEMIPTERA, REDUVIIDAE)

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(*Received* 15 *July* 1960)

(With Plate 1)

INTRODUCTION

The predatory habit is widespread in the Heteroptera; over half of the families whose biology is known live by the capture of living invertebrate prey.

All Hemiptera must ingest liquid food and as a consequence their salivary secretions play an important part in feeding. This is perhaps most clearly seen in the predatory Heteroptera where the saliva has assumed the function of a venom, and among these it is the assassin bugs of the Reduviidae that have most extensively exploited the habit. Their ability to paralyse their captures rapidly has long been known to students of natural history; Degeer (1773), writing of *Reduvius personatus*, observed: 'La Mouche une fois piquée mourut promptement, ce qui denote assez, que la Punaise dois sans doute verser dans la playe quelque venin, dont l'effet très actif.' Later writers have suggested the salivary glands (Miller, 1953), and the maxillary glands (Poisson, 1925) as the source of the venom, but its composition and mode of action have not been examined.

Two examples will suffice to illustrate the efficacy of assassin bug venom. The first instar larva of the harpactocorine reduviid *Rhinocoris carmelita* is able to paralyse within 10 sec. a final instar larva of *Ephestia kuhniella*, over 400 times its own weight. Again, the large reduviine *Platymeris rhadamanthus*, provided with a cockroach, *Periplaneta americana*, puts an end to the convulsive struggling of its prey within 3–5 sec., and abolishes the last flickering movements from its appendages within 15 sec. To do this it injects 10–12 mg. of saliva, and thereafter removes the products of external digestion, amounting to between 40 and 60% of the prey's live weight, at a rate of 1·5–2 mg/min.

It is the purpose of this paper to describe the action of assassin bug saliva on whole animals, selected organs and tissues, and to examine components of the saliva that play a part in paralysis and external digestion.

MATERIALS AND METHODS

(1) *Source of saliva*

Platymeris rhadamanthus saliva was obtained in quantity by exploiting the spitting behaviour of the species (Vanderplank, 1958; Edwards, 1960). For routine

collections, adults that had fed 3–5 days previously were placed under a 10 cm. diametrically broken Petri dish. The animal was molested by tapping its thorax with a seeker, through the break in the Petri dish, thus inducing it to spit saliva from its rostrum on to the glass above. After a series of animals had been extracted to exhaustion the saliva was allowed to dry and was then scraped from the surface with a steel blade and stored over $CaCl_2$. The effects of injecting solutions of saliva so collected, at appropriate concentrations (10–15%), had effects indistinguishable from natural predation, and it is assumed that the saliva secreted during spitting behaviour is the same as that injected into its prey. The term 'saliva solutions' refers to saliva powder freshly dissolved in boiled distilled water at concentrations of 1% and above, and in saline (Hoyle, 1953) at concentrations below 1%.

(2) *Pharmacological preparations*

The *Periplaneta* heart-dorsum provided a quickly made assay preparation. An adult male was pinned dorsal side down to a cork slab and all but the terga with the attached heart was dissected away. The 'bath' formed by the abdominal terga was washed with saline, then almost emptied, and placed in a damp chamber. It was used after regular beating resumed, usually within 20 min. of preparation. With occasional changes of saline this heart preparation would continue regular beating for over 16 hr.

Periplaneta also provided a central nerve cord preparation. After their heads had been removed, adult males were attached dorsal side up to a cork slab with cellulose tape. The abdominal terga and viscera were removed, and the ventral diaphragm gently torn. The nerve cord was then freed between the last and penultimate ganglia to allow the insertion beneath the cord of a pair of recording electrodes. The majority of tests were of short duration and were completed within 30 min. of dissection. The recording apparatus consisted of Tektronix type 122 and 53/54 C preamplifiers, and Tektronix type 520 cathode-ray oscilloscope. Test materials were delivered from a graduated pipette to the region of the last abdominal ganglion. A neon stimulator of variable frequency and voltage was used.

(3) *Electrophoresis*

(*a*) *Tiselius.* A bulk sample of 65 mg. of saliva powder collected over a period of 6 weeks from twenty adult *Platymeris* was dissolved in 2·5 ml. 0·1 M acetate buffer, pH 5·6, and dialysed against buffer overnight at 3° C. A small protein precipitate amounting to 2 mg. was removed by centrifugation. The remaining solution was subjected to Tiselius electrophoresis in a Perkin Elmer Model 38 apparatus.

(*b*) *Zone electrophoresis.* The starch gel method of Smithies (1955) was used with a discontinuous buffer system (Poulik, 1957). The gel was prepared with veronal buffer pH 8·6, and 0·3 M borate buffer pH 8·6 was used in the electrode tanks. Electrophoresis was carried out at room temperature for 16 hr., with a potential gradient of 6 V./cm. After electrophoresis the gel was stained with amidoschwarz.

(4) *Enzyme assay*

(*a*) *Protease.* The activity of the salivary protease was estimated by the method of Charney & Tomarelli (1947). Sulphanilamide azocasein was prepared from commercial 'light white' casein after de-fatting with acetone. 2% stock solutions were prepared by dissolving the azocasein powder in a small volume of 1% Na_2CO_3, neutralizing with 0·1 M-HCl and making up to volume with distilled water. Stock solution was stored under toluene and used within 6 days of preparation. The final buffer concentration in most experiments was 0·1 M phosphate or borate. Optical densities were read at 450 mμ with a Unicam SP. 600 spectrophotometer.

(*b*) *Hyaluronidase.* Preliminary tests were made using the 'ACRA' test of Burnet (1948), as modified by Evans, Perkins & Gaisford (1951). Viscosity reduction of synovial fluid was further examined using an Ostwald viscometer of about 0·5 ml. capacity and water-time of 14 sec. Synovial fluid collected from astragalo-tibial joints of newly slaughtered cattle was pooled and centrifuged at 8000 g. for 30 min. It was stored over chloroform at −15° C.

(*c*) *Lipase.* The methods of Cole (Baldwin & Bell, 1955) and Bier (1955) were used with olive oil emulsion and Tween 20 respectively as substrates.

(*d*) *Esterase.* The action of saliva on ethyl butyrate was examined by the method of Harrer & King (1941), and on acetylcholine by Glick's (1938) electrometric titration method.

(*e*) *Adenosine triphosphatase.* The method of Bailey (1942) was used.

(*f*) *Serotonin.* Serotonin was tested for by the method of Jepson & Stevens (1953).

RESULTS

Action of Platymeris *saliva on the whole animal*

The saliva proved to be toxic to a wide range of insects representing seven orders. Their own species are immune: application of 0·1 ml. of 1% *Platymeris* saliva to the heart and gut of a 5th instar *Platymeris* nymph after removal of the abdominal terga caused no marked alteration in their rhythmic contractions over a period of more than 3 hr. The toxicity of 1% saliva solutions toward the *Periplaneta* heart-dorsum preparation is not reduced by previous mixture with an equal volume of *Platymeris* haemolymph. The haemolymph alone is non-toxic, but the crop contents do preserve the toxicity of the saliva for at least 2 days.

Enteral and topical applications of saliva are not toxic to arthropods; the saliva must enter the haemocoel. *Periplaneta* drank 1% saliva solution without ill effect and *Calliphora* larvae were unharmed by 6 hr. immersion in 1% solution.

In the experience of the writer the bite of an assassin bug causes intense localized pain and swelling and leaves a long-standing necrotic pit. The dried saliva powder is an irritant of eye and nose membranes, causing oedema, vasodilatation, copious mucous secretion and respiratory disturbances similar to those caused by viper venom (Stanic, 1956).

The LD 50 of *Platymeris* saliva for *Periplaneta americana* was determined by

injecting a volume of saliva similar to that delivered in natural predation (*c.* 0·01 ml.) through the left prothoracic pleuron. Successive dilutions from a stock of 10% saliva were injected into groups of ten animals. The LD 50 for 18 hr. at 28° C. was 10·25 mg./kg.

Action of saliva on heart-dorsum preparation

Platymeris saliva at concentrations approximating to the natural concentration (5–15%) causes immediate and violent contraction and cessation in systole, together with general contracture of the tergal musculature. A slow relaxation of the inexcitable tissue follows after about 30 sec. and continues for 2–3 min. At a dilution of 10^{-1} (w/v) the action is as described above except that the relaxation phase is extended to between 5 and 10 min. At concentrations of 2×10^{-2} and below, an initial brief acceleration in rate of heartbeat precedes an irregular slowing, leading to cessation at between 30 and 70 sec. A lower limit to definite activity is reached at concentrations of about 10^{-6}. This concentration induces a slight increase in rate of beating in most preparations, followed by a decrease in rate and amplitude. The beat becomes irregular and contractility disappears after 10–20 min.

Extracts of both the anterior and posterior lobes of the principal salivary gland are toxic when applied alone, but the contents of the accessory salivary gland have no toxic action.

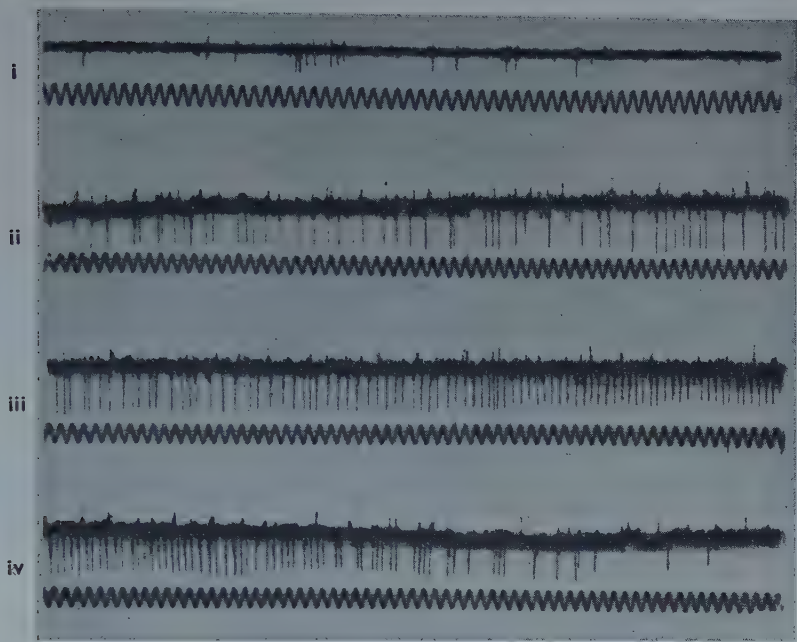
That potent toxicity is a special property of predatory bugs within the Heteroptera is demonstrated by the results shown in Table 1, where the action of homogenates of the salivary glands of a range of species is compared.

Table 1. *Toxicity of Hemipteran salivary gland homogenates*

Species	Quantity applied	Action
Platymeris rhadamanthus, *Rhinocoris carmelita*, *Reduvius personatus*	0·01 parts single gland in 0·1 ml. saline	Immediate cessation in systole. General contracture
Rhodnius prolixus, *Triatoma protracta*	Entire pair glands in 0·5 ml. saline	No action
Naucoris cimicoides	0·01 parts single gland in 0·1 ml. saline	Immediate cessation in systole. General contracture
Oncopeltus fasciatus, *Pentatoma rufipes*	Entire pair glands in 0·05 ml. saline	Slow decrease in amplitude. Slight increase in rate, cessation after some minutes

Action of saliva on nerve

Application of 0·1 ml. of 5–10% saliva solution in the region of the last abdominal ganglion causes within a few seconds an increase in electrical activity leading to intense repetitive discharge of giant fibres after 10–15 sec. The bout of intense activity terminates abruptly and thereafter the cord ceases to conduct; the post-synaptic response to electrical and mechanical stimulation of the cercal nerve is abolished. Excerpts from a typical record of the activity are shown in Text-fig. 1*a*. At lower concentrations the action is similar but the latent period preceding the activity is prolonged (Table 2).



Text-fig. 1 (*a*). Extracts from oscillograph records of the action of *Platymeris* saliva on the abdominal nerve cord of *Periplaneta americana*. (i) Record from last abdominal connective before application of saliva. (ii) and (iii) Onset of repetitive discharge after application of 0·1 ml. 1 % saliva in the region of the last abdominal ganglion. (iv) Decline of activity. Time marker: 50 c.s.

Table 2. *Latent period and duration of the discharge of* Periplaneta *nerve cord exposed to* Platymeris *saliva at various concentrations*

Concentration of saliva (%)	Latent period	Duration of discharge (sec.)
10	3–6 sec.	50–60
1	110–240 sec.	60–90
0·1	180–500 sec.	120–600
0·01	>45 min.	

Pricking the neural lamella under 0·01 % saliva produces a general discharge readily distinguishable from localized damage discharge. It appears that the neural lamella and/or perineurium provide an adequate barrier at low concentrations of saliva.

When a 0·1 % solution is applied to the cord, seven to nine separate successive bursts of repetitive activity could frequently be distinguished, each occupying 3–5 sec. In these cases the giant fibres were discharging singly. During the brief burst the frequency of discharge rose from 10 per sec. to a peak of *c.* 500 per sec. before becoming silent. At lower concentrations persistent repetitive discharge at various frequencies was frequently recorded (Text-fig. 1*b*).

The action of *Platymeris* saliva on a non-synaptic preparation was examined using a connective between the last thoracic and first abdominal ganglia of the locust *Schistocerca gregaria*. A length of connective was functionally isolated by crushing the cord adjacent to both ganglia until through conduction was abolished. The isolated connective was stimulated anteriorly and recordings were taken from the posterior end. 1 % saliva solution caused a decay in conduction beginning 80 sec. after application, and finally abolishing conduction after 120 sec.

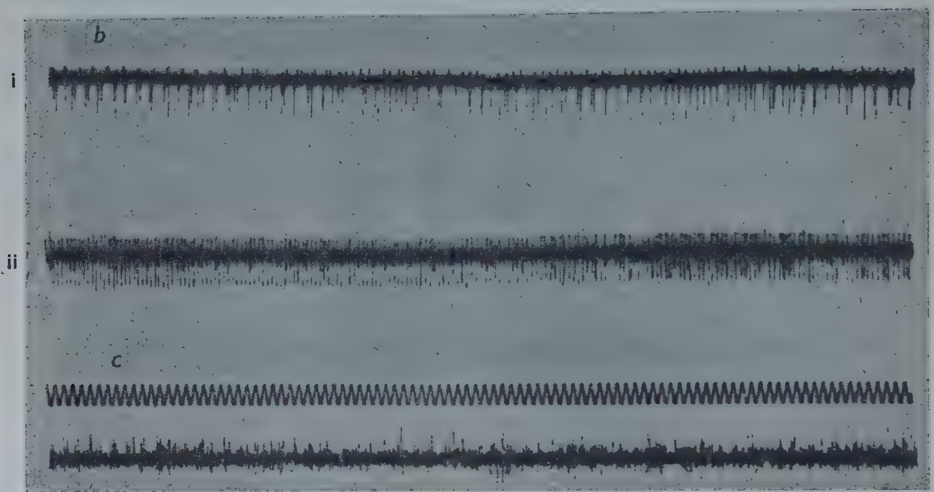


Fig. 1 (*b*). (i) and (ii) Typical oscillograph records of persistent repetitive discharge of *Periplaneta* nerve cord, induced by *Platymeris* saliva. (*c*) Action of bee venom on abdominal nerve cord of *Periplaneta*. For details see text. Time marker: 50 c.s.

Brief accelerating repetitive discharges comparable to those induced by *Platymeris* saliva were observed with a preparation of bee venom, (Text-fig. 1*c*) containing the contents of six poison sacs in 0·25 ml. saline.

Trypsin solution (1 % crystalline trypsin in saline) caused no increase in electrical activity within 30 min.

Action of saliva on muscle

Contraction of the tergal musculature in the heart-dorsum preparation has been noted above. The isolated Malpighian tubules of many insects exhibit rhythmic coiling and uncoiling effected by a spiral muscle band that is said to lack innervation (Palm, 1946). The action of *Platymeris* saliva on this muscle was examined using segments of the Malpighian tubules of *Schistocerca gregaria*. Segments were placed in a watch-glass containing 1 ml. saline (Belar, 1929, as used by Cameron, 1953). The addition of saliva solution was effected by briefly withdrawing the tubule with a small volume of saline into a wide-mouthed pipette. Saliva solution was added to the watch-glass and mixed to a known final concentration. The tubule segment was then returned. At concentrations down to 10^{-2} the immediate response was strong coiling followed by slow uncoiling with con-

current lysis of the tubule cells. At lower concentrations down to 10^{-6} the response was variable. In general the contractions became irregular and incomplete, movement eventually ceasing in either the coiled or extended state.

Lytic action of the saliva

Breakdown of fat body is the first visible effect of the saliva after paralysis. Sarcosomes of *Calliphora* flight muscle ruptured instantly when fragments of tissue mounted in saline under a coverslip were irrigated with a 0·1% solution. Muscle striation became diffuse within 5–10 min.

Gross changes in the appearance and mechanical properties of nervous tissue are observable within a short time of immersion in saliva solution. Segments of ventral nerve cord became opaque and lost rigidity. Previously clear outlines of giant fibres became diffuse and slight agitation reduced the contents to an homogeneous appearance. A remnant of the neural lamella which persisted after prolonged digestion appeared to consist largely of the collagenous components of the lamella (Smith & Wigglesworth, 1959). Histological examination of the action of the saliva on excised ganglia of *Oncopeltus fasciatus* using the osmic acid/ethyl gallate technique (Wigglesworth, 1957) revealed a rapid breakdown of cell membranes from the perineurium inwards, previously continuous lipid components of the cell walls becoming discontinuous and diffuse (Pl. 1, *a–c*). The neural lamella remained intact, although separation of laminae was observable after prolonged treatment.

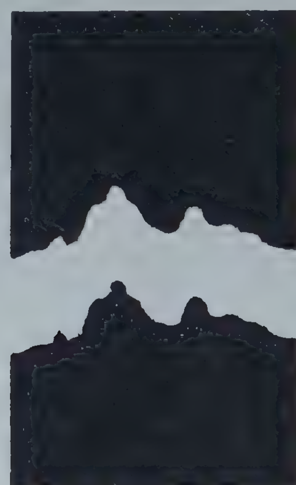
Composition of Platymerus *saliva*

The dried saliva is clear vitreous material, readily flaking to form a white powder. In the dried state it retains toxicity for at least 3 years, but declines slowly in potency in aqueous solution. Freshly secreted saliva has a pH of 6·6–6·8. Toxicity is retained after prolonged dialysis against distilled water in the cold and toxicity is not recoverable from concentrated dialysate. Toxicity is destroyed by papain and by performate oxidation by the method of Hirs (1956). Smears and sections gave negative tests for mucoid substances using the Bismark brown method of Leach (1947) as used by Day (1949) in examining the occurrence of mucoid substances in insects. Tests for mucoprotein using the Erlich direct reaction (Gottschalk, 1958) were also negative.

A single analysis of undialysed saliva gave the following percentage composition: C, 45·6%; H, 7·24%; N, 13·6%.

The ascending arm boundaries of Tiselius electrophoresis at pH 5·6 are shown in Text-fig. 2. The saliva is a complex mixture containing at least 6–8 proteins, the two major peaks having mobilities of $8{\cdot}4 \times 10^{-5}$ and 13×10^{-5}. It is of interest that the snake venoms studied electrophoretically by Poulsen, Joubert & Haig (1946) showed patterns of similar complexity. *Naja nigricollis*, the spitting cobra, had eight peaks, with mobilities between $11{\cdot}7 \times 10^{-5}$ and $0{\cdot}3 \times 10^{-5}$ at pH 6·2. Grasset, Brechnuhler, Schwartz & Pongratz (1956) found eight fractions in the venom of Russell's viper at pH 6.

Six fractions separated in starch gel at pH 8·5 (Text-fig. 3). Attempts were made to isolate a toxic fraction among the proteins separated by starch gel electrophoresis. Protein zones were located by staining a narrow strip from the margin of the run, replacing it as a marker so that the protein-bearing blocks could be cut from the gel. The contents were extracted by pressure after freezing and thawing the blocks. After dialysis against 0·2 M saline at pH 7 the extract was applied to a *Periplaneta* heart-dorsum preparation. Although the quantity of saliva used in the separation was sufficient to produce instantaneous effects had it been evenly distributed through the gel, none of the fractions had effects comparable to those of the whole saliva, nor had recombinations of the major bands.



Text-fig. 2. Tiselius electrophoresis of *Platymeris* saliva. Ascending arm, pH 5·6.

Text-fig. 3. Electrophoretic pattern of the saliva of *Platymeris rhadamanthus* in starch gel, pH 8·6. Horizontal hatching indicates intensity of staining. + indicates relative proteolytic activity.

Enzymes in Platymeris *saliva*

(*a*) *Protease*

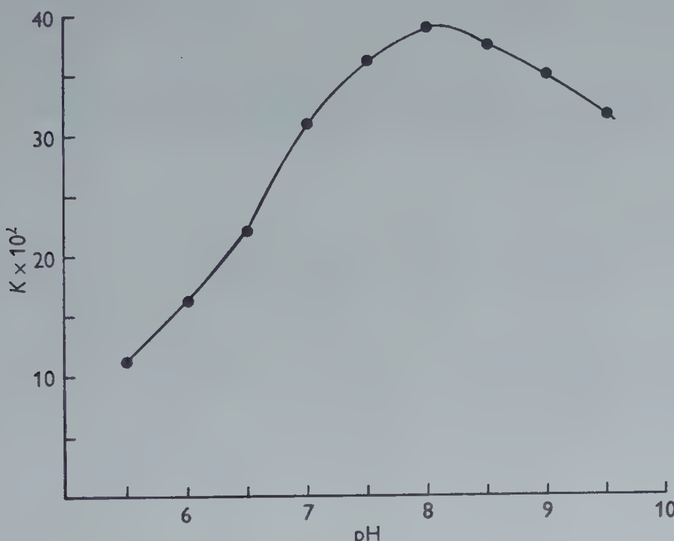
Three proteolytic zones were recognized in the electrophoretic pattern of *Platymeris* saliva using the method of Cuthbertson (personal communication) in which an agar gel containing casein, placed in contact with the starch gel, is cleared where it overlies a protease. The major protein zone proved to have strong proteolytic activity, while a neighbouring band and the extreme anode-migrating band, both containing less protein, showed much weaker activity (Text-fig. 3).

Both lobes of the principal salivary gland secrete protease as shown by the micro-method of Pickford & Doris (1934), but the contents of the accessory gland are not proteolytic.

Specificity. Beside casein and gelatin, *Platymeris* saliva digested blood fibrin and elastin. Release of colour from Congo red-stained substrate was maximal in the region of pH 8–8·5. Collagen, however, was not attacked; no evidence of activity was found using Hobson's (1931) method. The liberation of tyrosine from tendon estimated with Folin and Ciocalteau reagent was comparable to that of trypsin.

A preliminary examination of the major points of cleavage of the insulin B chain by *Platymeris* saliva indicates a similarity to a combination of trypsin and chymotrypsin.

pH *and activity.* Using the method of Charney & Tomarelli (1947) a broad pH optimum about pH 8·2 was found for a digestion period of 30 min. at 30° C. (Text-fig. 4).



Text-fig. 4. pH/activity for *Platymeris* salivary protease. Azocasein substrate.

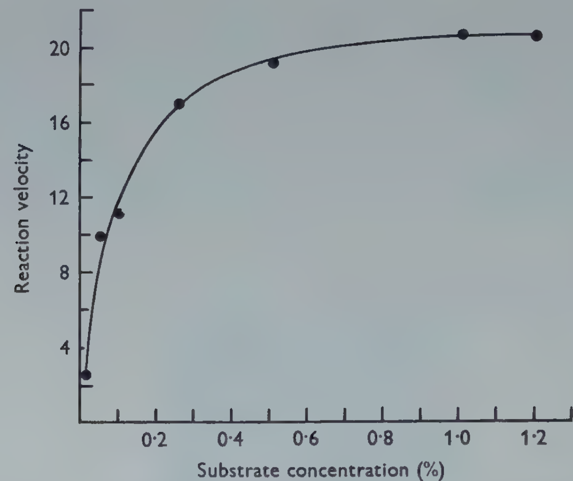
Substrate concentration. The substrate/activity curve for 30 min. digestion at 30° C. is shown in Text-fig. 5. A figure of 0·70 % azocasein for K_m. calculated from the results shows close similarity to that of *Calliphora* larval gut protease determined by Evans (1958) using the same substrate.

Temperature and activity. For a 30 min. reaction period at pH 8·2 a temperature optimum of 40° C. was found (Text-fig. 6).

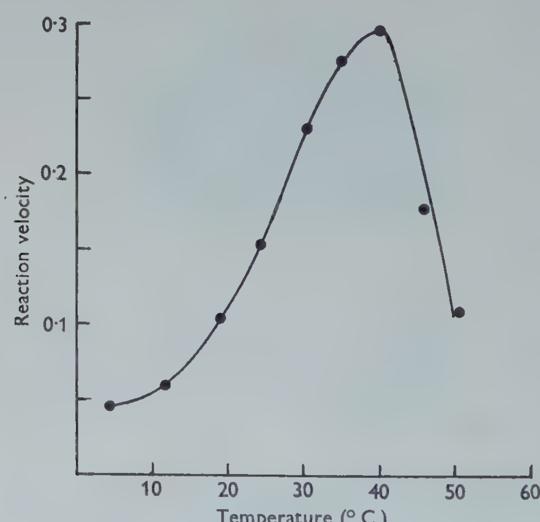
Inhibitors. (i) *Cyanide.* No inhibition was observed at pH 8·2 using 0·5 % casein solution and saliva at $0·5 \times 10^{-4}$ in cyanide solutions of 0·01, 0·05 and 0·1 M.

(ii) *Trypsin inhibitor.* The digestion of 0·5 % azocasein solution was examined using crystalline trypsin and *Platymeris* saliva at concentrations of $0·13 \times 10^{-3}$ with

and without equal quantities of soybean trypsin inhibitor. After 30 min. at 30° C., trypsin digestion was 91·5% inhibited and *Platymeris* salivary protease 15·5% inhibited.



Text-fig. 5. Substrate concentration/activity curve for *Platymeris* salivary protease. Azocasein substrate.

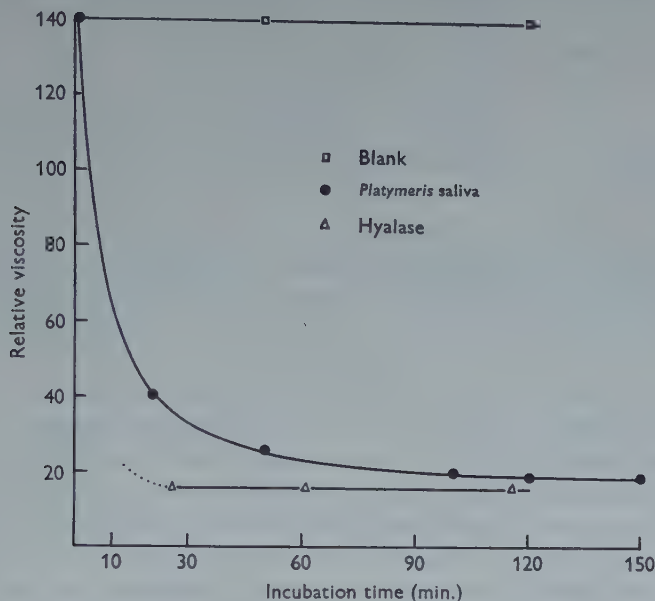


Text-fig. 6. Temperature/activity curve for *Platymeris* salivary protease. Azocasein substrate.

(*b*) *Hyaluronidase*

The reduction in viscosity of synovial fluid by *Platymeris* saliva shown by the 'ACRA' test was further examined by means of an Ostwald viscometer as follows. Synovial fluid was diluted 1:1 with isotonic saline. Hyaluronidase (Benger 'Hyalase') and *Platymeris* saliva were dissolved in saline to give solutions containing 0·5 mg./ml. Saliva solution heated to 98° C. for 10 min. provided a blank.

2 ml. synovial fluid was incubated at 30° C. with 0·25 ml. enzyme solution and the relative viscosity determined in an Ostwald viscometer in the same water-bath. Text-fig. 7 shows the fall in relative viscosity caused by hyaluronidase and by saliva.



Text-fig. 7. Reduction of viscosity of synovial fluid by *Platymeris* saliva.

(*c*) *Lipase and esterase*

Negative results were obtained in all experiments on *Platymeris* saliva. Lipase activity was found in the extracts of the mid-gut wall. Preliminary experiments indicated a tenfold increase in lipase activity in the gut wall 2 hr. after feeding compared with that of animals starved for 20 days.

(*d*) *Phospholipase*

The lytic activity of *Platymeris* saliva suggested the presence of a phospholipase, one of the most widespread constituents of animal venoms (Zeller, 1951). Phospholipase activity as measured by alkaline titration of an unbuffered emulsion of commercial lecithin (B.D.H.) was negligible. Through the generosity of Dr R. M. C. Dawson the action of *Platymeris* saliva on ^{32}P-labelled lecithin was examined. Saliva was incubated with an unbuffered emulsion containing ^{32}P-labelled lecithin for 30 min. at 37° C., and the acid-soluble phosphate released was compared with the total acid-soluble ^{32}P in the sample released by oxidation with $HClO_4$ (Table 3).

Table 3. *Release of acid-soluble* ^{32}P *from lecithin by* Platymeris *saliva*

	Blank	*Platymeris* saliva	Total ^{32}P
Activity counts/min.	64	1135	6478
% of total	1	17·5	—

(e) *Adenosine triphosphatase*

The quantity of acid-soluble phosphate liberated from 0·005 M-ATP solution plus 0·1 M-KCl with histidine buffer pH 7 did not exceed the blank value (Table 4).

Table 4. *Release of acid-soluble phosphate from ATP by* Platymeris *saliva*

	Blank no saliva	Without activator	Activator 0·005 M-Ca	Activator 0·005 M-Mg
P liberated	11·5	10·5	11	11

(f) *Serotonin*

Negative tests were obtained from spots of *Platymeris* saliva containing 1 mg. in a test for which a sensitivity of 10^{-4} μmoles cm.^{2} is reported (Jepson & Stevens, 1953).

DISCUSSION

Platymeris saliva, a mixture of at least six proteins, is comparable in complexity to snake venoms similarly studied. Three electrophoretic fractions, one of them the major component of the saliva, are proteolytic. Patterson & Fiske (1958) also reported the presence of three proteolytic fractions in gut homogenates from the stable fly *Stomoxys calcitrans*, though their electrophoretic behaviour differs from that of *Platymeris* saliva. The proteolytic activity of whole saliva resembles that of gut extracts from other insects examined with an azocasein substrate. The substrate affinity of 0·70% is closely similar to that of the gut protease of larval *Calliphora erythrocephala* (Evans, 1958). The pH optimum also resembles that of the *Calliphora* protease (7·8) and of *Blatella germanica* (8·2) (Day & Powning, 1949). Powning, Day & Irzykiewicz (1951) have examined the degree of inhibition of protease extracts from the gut of several species including Orthoptera, Diptera, Lepidoptera and Coleoptera, using an azocasein substrate. Only *Tenebrio* protease resembled that of *Platymeris* in being less inhibited than trypsin. The specificity of the whole saliva resembles that of trypsin plus chymotrypsin, although it also shows elastase activity.

The widespread presence of hyaluronidase as a 'spreading factor' in animal venoms has been reviewed by Favilli (1956). Romanini (1949) reported mucinolytic activity in whole insect extracts of several Heteroptera. The present study is the first demonstration of hyaluronidase activity in the pure saliva of an insect, although Stevens (1956) found activity in salivary gland and gut homogenates of *Periplaneta americana* using a turbidometric technique, confirmed in this study viscosimetrically using the contents of the *Periplaneta* salivary reservoir. Since Ogston & Sherman (1959) have demonstrated that trypsin and chymotrypsin together can remove 65% of the protein from the hyaluronate-protein complex of synovial fluid without loss of viscosity, *Platymeris* salivary protease is unlikely to be involved in the viscosity-reducing activity of the saliva. Hyaluronidase is said to cause the separation of the epithelial cells of the insect midgut (Day, 1949). In

the present study separation of dipteran fat body cells, except where they are in contact with oenocytes, was effected by immersion in saline containing 0·1% hyaluronidase (Benger Hyalase). It appears that in the insect as well as in the vertebrate hyaluronidase will facilitate the penetration of active substances by attacking the intercellular matrix, thus acting as a 'spreading factor'.

Platymeris *saliva as a venom*

The responses of innervated and non-innervated muscle, intact and isolated nerve, to treatment with *Platymeris* saliva indicate that the mechanism of paralysis does not involve a specific site of action. Rather it seems, the saliva attacks and disrupts the cell membranes on which the functioning of excitable tissue depends. It is suggested that the subsequent lysis is a gross extension of the initial membrane breakdown that causes paralysis, or to put the process in its adaptive context, that paralysis by assassin bug saliva is a special function of external digestion.

Knowledge of insect venoms is almost entirely restricted to those of the Hymenoptera. Pavan's (1958) review of insect venoms does not deal with the Heteroptera. The study of *Habrobracon* venom by Beard (1952) is the only work on the action of an insect venom on an insect, with the exception of a short note by Hartzell (1935) on ganglion lesions in a cicada caused by wasp venom. *Habrobracon* venom however is prey-specific, is not proteolytic and appears to act as an 'insect curare' and therefore stands in contrast to *Platymeris* saliva.

A parallel to *Platymeris* saliva outside the Insecta is to be found in the venom of snakes, also a salivary secretion. In both, proteins act as toxins, both have enzymic activity, and cobra and viper venoms show a similarity in the number of proteins present. The fate of the vertebrate and arthropod prey, however, share less ground, since only in the vertebrate can the haemolytic and histamine shock reactions play an important part in paralysis. Rapid loss of conduction following intense central nervous activity, and general contraction followed by relaxation and inexcitability of muscle are the responses of the arthropod for which the proteins of the assassin bug's saliva are responsible.

Tobias (1955) has demonstrated that hyaluronidase, papain, chymotrypsin and collagenase have no effect on the conduction and trans-surface potentials of isolated lobster axons. Nor does trypsin influence the activity of the insect central nervous system; and, unlike *Platymeris* saliva, proteases are slow to lyse erythrocytes (Ballentine & Parpart, 1940). Protease will, however, cause contraction and depolarization of frog sartorius muscle (Tobias, 1955) and bring the cockroach heart to systolic standstill.

Phospholipases cause both depolarization of nerve, and contraction and depolarization of muscle, and Tobias concludes that the integrity of the phospholipid layers is indispensable for their normal functioning. Now, Richards & Cutkomp (1945) state that cobra venom, which contains phospholipase A, is 'quite toxic to insects' causing paralysis of the nervous system, but that injections of 'maximal doses of lysolecithin into the haemocoel of insects was without effect'. They observe that the lysolecithin may not have penetrated to the nerves, but conclude that the

lysolecithin is not toxic to insects. Cobra venom contains protease as well as phospholipase and this will have effected muscular paralysis. Further, Tobias has shown that lysolecithin acts on lobster axons, although the ionic environment influences the degree of activity. The action of phospholipase C shows that the formation of lysophosphatides is not necessary for damage to occur. Bee venom contains phospholipase and its action on the insect central nervous system resembles that of *Platymeris* saliva, but it is a complex mixture (Neumann & Haberman, 1956) and can yield no precise comparison with *Platymeris* saliva.

The toxic action of the saliva cannot depend on hyaluronidase and protease alone, though these will facilitate its entry into tissue. The absence of the commonly occurring venom components cholinesterase, ATP-ase and serotonin and the rapid lysis (particularly of erythrocytes and mitochondria) effected by the saliva suggest that the action depends on a phospholipid-disrupting enzyme. The phospholipolytic activity of the saliva was found to be weak compared with snake venom, but as Lovern (1955) comments: 'If in the tissues lipids are lightly bound into macromolecules, their physical and chemical properties, including their behaviour as enzyme substrates, are likely to be different from those of free lipids, e.g. as a result of orientation and of changes of solubility'.

The role of Platymeris *saliva in digestion*

The saliva injected into the prey produces a viscid fluid with lipid droplets dispersed through it. Proteins are digested externally, but lipid is not hydrolysed until the ingested material reaches the midgut where lipase activity rises after feeding has taken place. The protease activity of the gut wall is relatively low and does not appear to fluctuate with feeding. Since only tryptic digestion seems essential for protein absorption (Fisher, 1954) it is possible that the protein breakdown effected by the saliva during external digestion and subsequent storage in the capacious midgut crop of the assassin bug is sufficient to yield assimilable products.

SUMMARY

1. The responses of whole insects, selected organs, and tissues to treatment with the saliva of an assassin bug *Platymeris rhadamanthus* are described. The excitability of muscle and nerve is rapidly abolished.

2. In the general lysis that follows immobilization only cuticular and collagenous structures are spared. The disruption of lipid layers in the walls of nervous tissue is histologically demonstrable at an early stage.

3. The saliva contains at least six proteins, and lacks mucoprotein or other mucoid substance. Three proteolytic fractions were recognized after starch-gel electrophoresis at pH 8·6, one of them forming the major component of the saliva. Attempts to locate a toxic fraction were unsuccessful.

4. The alkaline endopeptidase activity of whole saliva characterized with an azocasein substrate closely resembles gut proteases of other insects examined with the same substrate.

5. Hyaluronidase is present in the saliva and with protease acts as a spreading factor by breaking down the intercellular matrix.

6. Lipase and esterase activity were not detected in the saliva, but gut-wall extracts were lipolytic.

7. The saliva shows weak phospholipase activity. ATP-ase, and serotonin were not detected.

8. The mode of action of assassin bug saliva as a venom and in external digestion is discussed. It is suggested that its toxicity is due to the disruption of phospholipid layers of the cell wall and is the first manifestation of general lysis during external digestion.

I am most grateful to Prof. Wigglesworth, my supervisor during this study, for his guidance and encouragement, to Dr F. L. Vanderplank, whose ready co-operation in sending *Platymerus* from Zanzibar made the study possible, and to Mr M. W. Rees for much help in protein matters.

I also wish to acknowledge the aid of Dr R. M. C. Dawson in enabling work on phospholipase, and Mr J. Heslop with whom first tests on the electrophysiological action of the saliva were made at the the Pest Infestation Laboratory, Slough.

The work was carried out during the tenure of a Rhondda Research Studentship at Gonville and Caius College, Cambridge.

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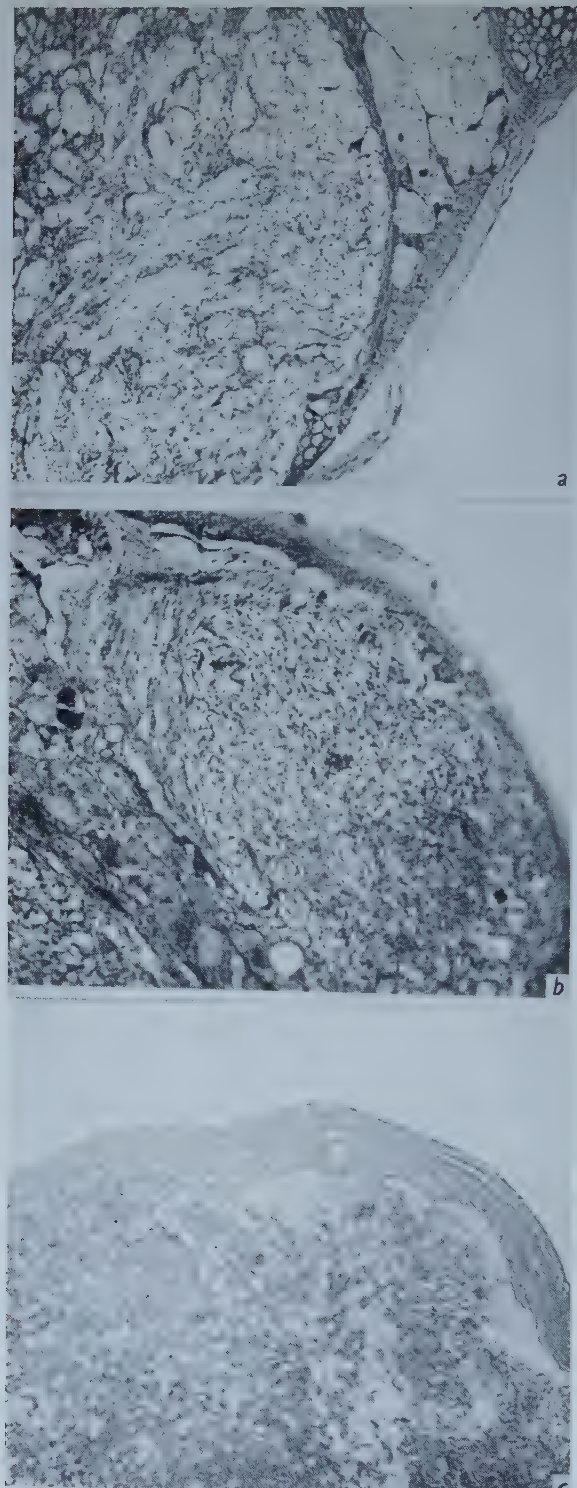

EDWARDS—THE ACTION AND COMPOSITION OF THE SALIVA OF AN ASSASSIN BUG *PLATYMERIS RHADAMANTHUS* GAERST. (HEMIPTERA, REDUVIIDAE)

(*Facing p.* 77)

Wigglesworth, V. B. (1957). The use of osmium in the fixation and staining of insects. *Proc. Roy. Soc.* B, **147**, 185–99.

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EXPLANATION OF PLATE

Stages in destruction of thoracic ganglion of *Oncopeltus fasciatus*. Transverse sections, 2 μ, osmic/gallate. *a*, 10 sec., histology normal. *b*, 10 min. lysis of outer axons visible, glial cells and inner regions intact. *c*, 30 min., lysis complete.

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With 4 text-figures
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THE RESPIRATORY CENTRE IN THE TENCH (*TINCA TINCA* L.)

II. RESPIRATORY NEURONAL ACTIVITY IN THE MEDULLA OBLONGATA

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(*Received 20 July* 1960)

INTRODUCTION

In a previous paper (Shelton, 1959) the effects of brain transections on the breathing movements of the tench were described. These experiments showed that a region of the medulla oblongata, extending from the posterior border of the facial lobe up to the level where the Vth and VIIth cranial nerves emerge, had to be intact for normal breathing movements to be produced. In the experiments to be described in this paper, the medulla was explored with needle electrodes in order to determine the distribution of neurones whose activity could be correlated with the breathing movements of the fish. Although they did not make an exhaustive search, Woldring & Dirken (1951) recorded volleys of spike potentials in rhythm with the movements of respiration in the medulla of the carp. They attributed this activity to two strips of tissue to the right and left of the midline at the anterior border of the facial lobe, and suggested that the discharges came from the motor neurones of the VIIth, IXth and Xth cranial nerves. Similar respiratory volleys have been recorded by Hukuhara & Okada (1956) in the carp and the catfish. These volleys were apparently obtained from a single electrode site in both species of fish. Hukuhara & Okada went on to show that the rhythmic discharges produced by the neurones at the active region continued when the medulla was isolated by cutting all the cranial nerves and transecting the midbrain and spinal cord. No work described so far has involved a detailed examination of the fish medulla, and information about the arrangement of respiratory neurones is very limited.

Since the pioneering work of Gesell, Bricker & Magee (1936), many workers have used recording electrodes to explore the medulla of mammalian preparations for respiratory neurones (Dirken & Woldring, 1951; Amoroso, Bainbridge, Bell, Lawn & Rosenberg, 1951; von Baumgarten, von Baumgarten & Schaefer, 1957; Haber, Kohn, Ngai, Holaday & Wang, 1957; Salmoiraghi & Burns, 1960). In spite of the considerable volume of work that has been done, using stimulation and transection as well as recording techniques, there is not complete agreement about the site and arrangement of respiratory cells within the mammalian brain stem. Several of the

* Present address.

differences are undoubtedly due to characteristics of the technique used. Some features of the recording technique (particularly *vis-à-vis* that of electrical stimulation) have emerged from the work on mammals and the ones relevant to the present work will be considered briefly. The recording electrode detects activity in a very localized region around the non-insulated tip and so is very selective, locating active units with considerable accuracy. Respiratory neurones are hard to find with recording electrodes and so are probably loosely scattered through the medulla. This dispersion of individual respiratory units is not revealed to the same extent by stimulation techniques and this is due, in part, to the spread of stimulus around the tip of the electrode. The spread will vary according to the stimulus parameters but inevitably a considerable number of units must be excited at any locus and the chances of affecting some part of even a dispersed system will be quite high. The indiscriminate excitation of a considerable number of neurones, probably involved in the co-ordination of different physiological activities, is an undesirable property of the technique which may complicate interpretation of the results (Amoroso *et al.* 1951; Liljestrand, 1953). Another reason for the recording technique showing a less widespread distribution of respiratory neurones is that some elements may not be detected by this method even though their stimulation may cause modification of the breathing movements. It is generally agreed that fine needle electrodes of the type usually used will detect activity from the neighbourhood of cell bodies and not from nerve fibres. Consequently, impulses in afferent and efferent pathways would not be recorded, whereas stimulation of these areas via similar electrodes would, presumably, be effective. This argument may be extended to include neurones which may operate in respiratory co-ordination only when the breathing is much modified, or even to generalized reticular units which never operate solely in respiratory co-ordination (Liljestrand, 1953). The stimulation method could include such units as part of the general respiratory system, although they would not be detected by recording techniques in the normally breathing animal.

Though all the techniques used in locating the neurones of the respiratory centre give valuable information, the detection of active sites by means of recording electrodes seems potentially more accurate and selective than other methods. It probably gives little or no information about activity in fibre pathways but this is hardly a serious limitation. A more significant fault of the method is that only those neurones which produce bursts of activity in rhythm with the breathing movements can be defined as participating in the co-ordination of these movements. It is possible that neurones producing different types of activity, such as a continuous discharge, may have a significant part to play in the generation and regulation of the respiratory rhythm. Though it is possible to think of ways in which the respiratory function of this hypothetical type of neurone could be detected, it is clearly not possible to test every active neurone found in the medulla for such a function. In the present paper, therefore, only those neurones which are active in rhythmic bursts are considered to be part of the respiratory centre.

METHODS

Tench from 45 to 70 g. in weight were deeply anaesthetized in 0·5–1·0% urethane solution, and a hole was cut in the top of the skull over the region of the medulla in which the search was to be made. The fish were fixed in the holder described previously (Shelton, 1959) and then were allowed to recover to a lighter level of anaesthesia which was maintained for the duration of the experiment. The urethane concentration for this was about 0·2%, although there was considerable individual variation and the level was arrived at by trial and error. With very light anaesthesia, rhythmic swimming movements were produced by the fish and occasionally the trunk and breathing rhythms were synchronized. This synchrony has been described in the goldfish by von Holst, and attributed to the rhythmical activity of a single automatic system in the medulla or spinal cord (von Holst, 1934*a*, *b*). In the present work complications due to the spread and synchronization of rhythmic activity were undesirable and so the concentration of urethane was increased to the point where the swimming movements became irregular or ceased altogether. During an experiment the fish were allowed to breath normally in about 1000 ml. of water which was continuously aerated. The water-level in the experimental tank was adjusted so that it came just below the hole in the skull, thus avoiding any problems of protecting the brain from osmotic and ionic stresses. In general, no physiological saline was necessary as the brain was bathed in a pool of body fluid which accumulated in the well formed by the cranium. Survival of the animals during the experiments was good and, after successful preliminary operations with little haemorrhage, there were no signs of deterioration. The experiments were done at room temperature.

The medulla was searched systematically using fine needle electrodes held in a manipulator which permitted calibrated movement in three planes. The electrodes were made from 25 μ diameter platinum wire which was insulated except at the tip by means of varnish or glass. The glass insulation gave a more durable electrode and was most frequently used, the over-all diameter of a unipolar electrode of this type being 40–60 μ. Bipolar electrodes were tried in some experiments, but localization of activity, as determined by changes in the discharge pattern produced by slight movements of the electrode, appeared to be the same with both types. The majority of the experiments were done with the simpler unipolar electrode. The signals were amplified in a Grass P4 pre-amplifier and displayed on one beam of a double-beam Cossor oscillograph. A loudspeaker unit was used to monitor the activity picked up. The breathing movements of the fish were displayed on the other beam of the oscillograph by means of a simple mechano-electric transducer attached to the operculum.

The electrodes were inserted perpendicularly into the brain, the distance of any recording site being measured from fixed reference points on the surface of the brain by means of the calibrated slides of the manipulator. These measurements on the three co-ordinates of the manipulator gave an approximate idea of the position of the electrode tip in the medulla and the loci from which respiratory activity was

obtained were confirmed later when the brain was sectioned. The brains were fixed in 10% formol in Young's teleost Ringer; sections were cut at 12–15 μ and stained in Heidenhain's iron haematoxylin. The electrode tracks were usually discernible in these sections and allowed identification of the active region. In many experiments the electrode was lowered further into the brain after an active site had been found, and in these cases the depth measurement on the manipulator had to be used for location. Measurements of the brain before fixation and after sectioning were used to correct the depth readings for the shrinkage which occurred. An electro-cautery device was also used in experiments on fifteen of the fish examined. The cauterizing current was applied through the electrode when this was in an active region and so produced a small burn in the brain tissue. A difficulty with this method was that the size of the burn varied in different electrode sites even though the output of the cautery was kept constant. In some cases there was spread of the burn back up the stem of the electrode. The breathing movements of the fish used in the cautery experiments were examined carefully for any effects caused by the destruction of active respiratory regions. In general, the methods described above made it possible to assign activity to particular regions within the medulla, although it is a little difficult to decide with what accuracy. Errors of 0·2–0·3 mm. may have been made in the location of some sites but in the majority of cases, where electrode tracks could be followed, the error was less than this.

Some anatomical studies were made on sections of the tench brain which had been sectioned transversely or longitudinally and stained by the silver techniques of Holmes (1947) or Romanes (1950).

RESULTS

The experiments were performed on forty-four tench, exploration of the brain being carried out from the level of the obex to the front of the cerebellum. In all the experiments, a total of 470 insertions of the electrodes was made from the dorsal surface of the brain. Several different types of activity were found, the most common being discharges which were not obviously related to any activity of the animal or to any stimulus that it was receiving. Spike discharges were found frequently in the vestibular nuclei and the crista cerebellis when vibrational stimuli of various kinds were given. Injury discharges were recognizable by their short duration and high frequency and great care had to be exercised in lowering the electrode through the nervous tissue if this type of discharge was to be avoided. In eighty-nine of the 470 electrode insertions made, it was possible to detect potential discharges, in rhythm with the breathing movements, from some point on the track of the electrode through the brain. In the fish, where the principal musculature involved in the breathing movements is that of the head region, there is clearly a danger that rhythmic activity of this sort detected in the brain may be due to movement artifacts. Movements of the brain with respect to the electrode tip could cause a periodic injury discharge in an otherwise inactive neurone or could bring a continuously discharging neurone within range of the recording system

during a certain phase of the breathing cycle. Every precaution was taken to avoid such artifacts; the skull was held immobile in the clamp and the discharges were examined for characteristics, such as a gradual increase in a unit's size or the eventual loss of a unit in a typical injury discharge, which might be expected if they were due to a movement of the brain.

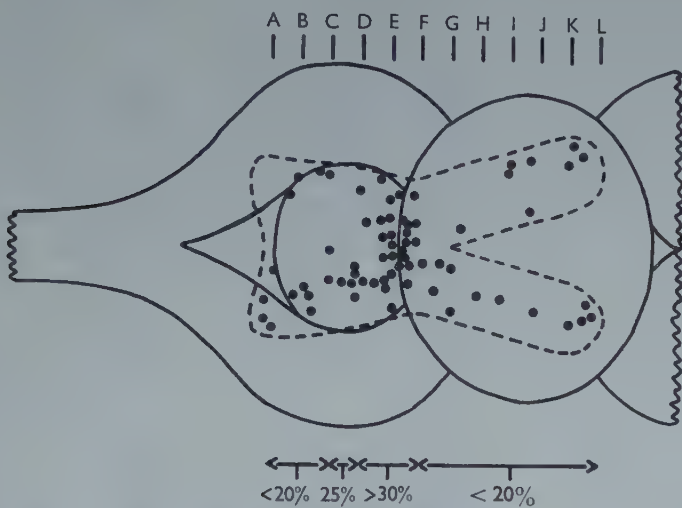

Fig. 1. Dorsal view of the tench medulla showing the positions of successful electrode insertions. The broken line delimits the proposed respiratory area and the percentages of electrode insertions which were successful at various levels within this area are indicated.

Localization of the respiratory neurones

The regions on the dorsal surface of the medulla in which successful insertions of the electrodes were made are plotted in Fig. 1. On the diagram there is clearly a higher density of active sites around the posterior border of the cerebellum and the front of the facial lobe than there is elsewhere. This is due in part to a relatively greater number of electrode insertions being made in this region during the searches, but this is not the complete explanation. If the successful insertions are expressed as percentages of the total number of insertions made in particular regions then differences are still found. The dotted line in Fig. 1 delimits the proposed respiratory area of the medulla within which the rhythmic activity was found. The area was divided transversely into eleven regions at the levels shown by the lines A to L. In the regions between lines D to F, over 30 % of the electrode insertions were successful in detecting respiratory activity, whereas anterior and posterior to these regions the successes fell to below 20 %. It seems likely therefore that the respiratory neurones are more densely arranged in this central area. This is not to suggest that even here large groups of respiratory neurones occur, forming a more or less discrete nucleus. At no point was it possible to make an electrode insertion with the certain knowledge that respiratory activity would be detected.

In Fig. 2 the loci within the medulla from which the respiratory discharges were obtained are plotted on a series of twelve cross-sections. These sections are taken at the levels A to L in Fig. 1. In the more posterior regions (sections A to C) the respiratory activity was detected very largely in the scattered reticular cells below

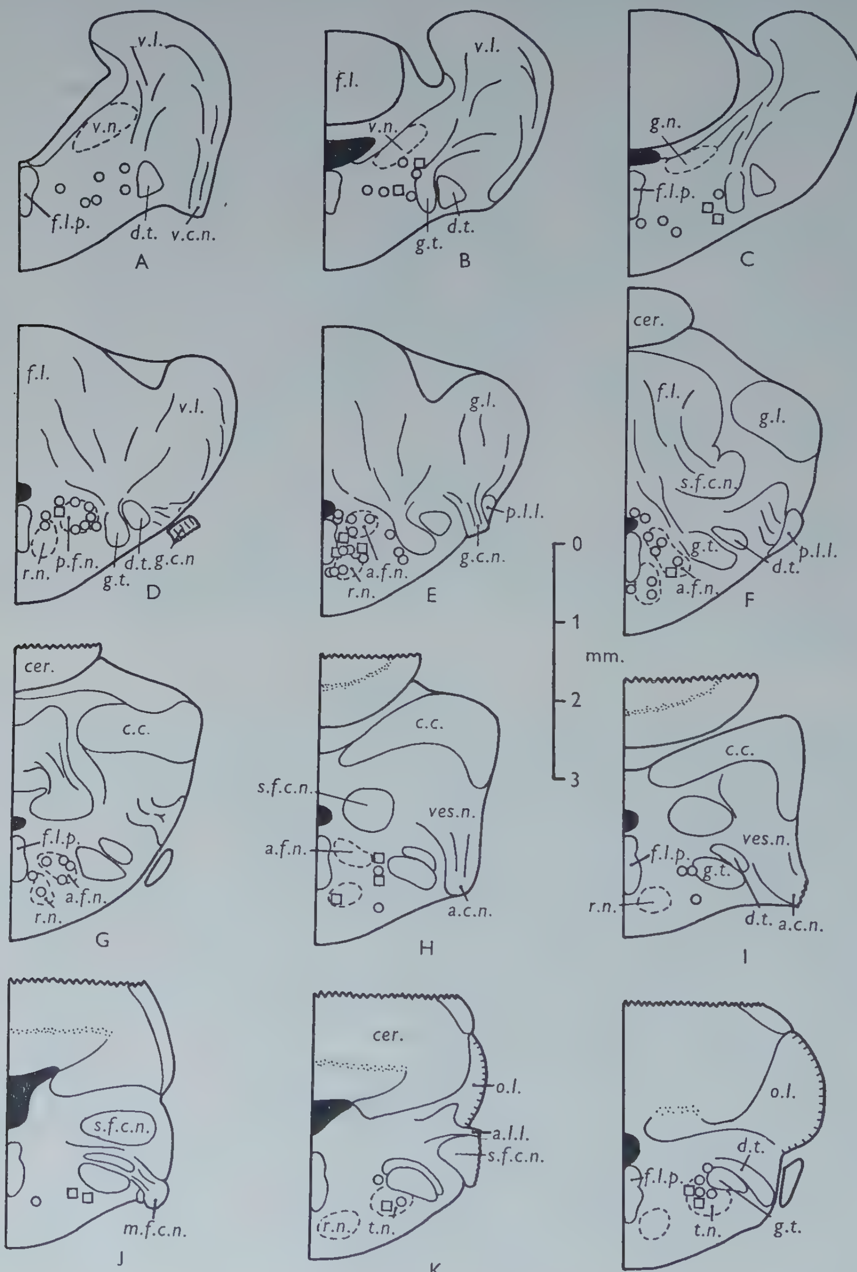

Fig 2. For legend see opposite.

the Xth motor nucleus. It is thought that none of the rhythmic discharges was obtained from neurones actually within the motor nuclei of the IXth and Xth cranial nerves, though it is difficult to be absolutely certain of this. The majority of active sites are certainly too deep to be associated with these nuclei, but one or two borderline sites occur where the motor nuclei are merging into the undifferentiated parts of the grey matter. At a somewhat higher level (section D) respiratory activity was quite definitely picked up from motor neurones as well as from reticular cells. The IXth and Xth motor nuclei, together with the posterior motor nucleus of the VIIth cranial nerve, form a continuous visceral efferent column extending from behind the obex almost to the point of emergence of the IXth cranial nerve (Kappers, Huber & Crosby, 1936). Rhythmic activity was detected only in those motor neurones situated at the front end of this column, which were therefore almost certainly part of the posterior facial nucleus. Kappers *et al.* suggest that this part of the facial motor nucleus is concerned with the innervation of some gill apparatus muscles such as the levator and adductor operculi. These muscles play an important part in the breathing movements of the tench (Shelton, unpublished). Respiratory discharges also occurred in the anterior facial motor nucleus (sections E, F and G) as well as in the reticular cells which in this region forms a fairly discrete nucleus situated ventrolaterally to the fasciculus longitudinalis posterior. Active units were found scattered in the grey matter at still higher levels, though the frequency of their occurrence decreased. The most anterior region from which respiratory discharges were obtained was the motor nucleus of the Vth cranial nerve (sections K and L).

Fig. 2. Transverse sections through the tench medulla at the levels A to L shown on Fig. 1. The regions in which bursts of activity coincided with the breathing movements are plotted; squares indicate activity coinciding with the opening (expansion) phase of the breathing cycle, circles activity coinciding with the closing (contraction) phase. Key to lettering:

a.c.n.	auditory (VIIIth) cranial nerve
a.f.n.	anterior facial (VIIth) motor nucleus
a.l.l.	anterior component of the lateral line nerve
cer.	cerebellum
c.c.	crista cerebellis
d.t.	descending root of trigeminal (Vth) nerve
f.l.	facial lobe
f.l.p.	fasciculous longitudinalis posterior
g.c.n.	glossopharyngeal (IXth) cranial nerve
g.l.	glossopharyngeal (IXth) lobe.
g.n.	glossopharyngeal (IXth) motor nucleus
g.t.	secondary gustatory tract.
m.f.c.n.	motor component of the facial (VIIth) cranial nerve
o.l.	optic lobe
p.f.n.	posterior facial (VIIth) motor nucleus
p.l.l.	posterior component of the lateral line nerve
r.n.	reticular nucleus
s.f.c.n.	sensory component of the facial (VIIth) cranial nerve
t.n.	trigeminal (Vth) motor nucleus
v.c.n.	vagal (Xth) cranial nerve
ves.n.	vestibular nuclei
v.l.	vagal lobe
v.n.	vagal (Xth) motor nucleus

Destruction of active sites

In experiments on fifteen tench, a number of active sites were destroyed by cautery after they had been detected by the recording apparatus. The average number of sites destroyed was just over three in each fish, and the highest number in any individual was eight. The destruction was on a very small scale therefore, and the conclusions which can be drawn are limited. The searches during these cautery experiments were arranged so that the regions destroyed were distributed over the whole respiratory area. In none of the fish were the breathing movements stopped completely as a result of the destruction of either a single or several sites. However, it was quite common for the movements to stop immediately a region was destroyed and for 2 or 3 min. to be taken for recovery. In many cases the pattern of breathing was different upon resumption although the changes were small ones such as slight variations in the breathing frequency. On other occasions, the effect was on some component of the pumping mechanism rather than on the breathing rhythm as a whole. Such things as the failure of one opercular flap to close effectively and distortion of the mouth during the complete breathing cycle were observed. As might be expected, some of these local failures were due to destruction of parts of the Vth or VIIth motor nuclei, although similar effects were obtained by destruction of other regions. On two occasions, for example, it was found that damage in the grey matter beneath the Xth motor nucleus caused some malfunction in the operculum of the same side.

Characteristics of the activity from respiratory neurones

In spite of the difficulty experienced in locating them, the respiratory neurones were not usually dispersed to the extent of occurring singly in the nervous tissue of the medulla. When an active site was located it was common to find that the discharges consisted of more than one unit (Figs. 3, 4) and that slight movements of the electrode at the active site would bring in different rhythmically firing units. In Fig. 3*a* there are records obtained from points 0·05 mm. apart in nervous tissue below the anterior facial motor nucleus, between it and the reticular nucleus. Lowering the electrode through the brain by this distance brought in a new unit towards the end of the original discharge which was then detected less definitely. A similar effect is seen in the records of Fig. 3*b*, obtained from the trigeminal motor nucleus. In this case the unit introduced by lowering the electrode by 0·08 mm. fired approximately in the intervals between bursts from the original unit.

Though the rhythmic pattern of activity from any one respiratory neurone remained fairly constant for long periods, there were considerable differences in the patterns obtained from different neurones. The duration of the rhythmic bursts in relation to a complete respiratory cycle, the frequency of the spike discharges within the bursts, and the phase of the respiratory cycle in which the burst occurred were all quite variable from neurone to neurone. Differences occurred in these features of activity obtained when the electrodes were in similar regions of the medulla in different animals or even in regions very close to one another in the

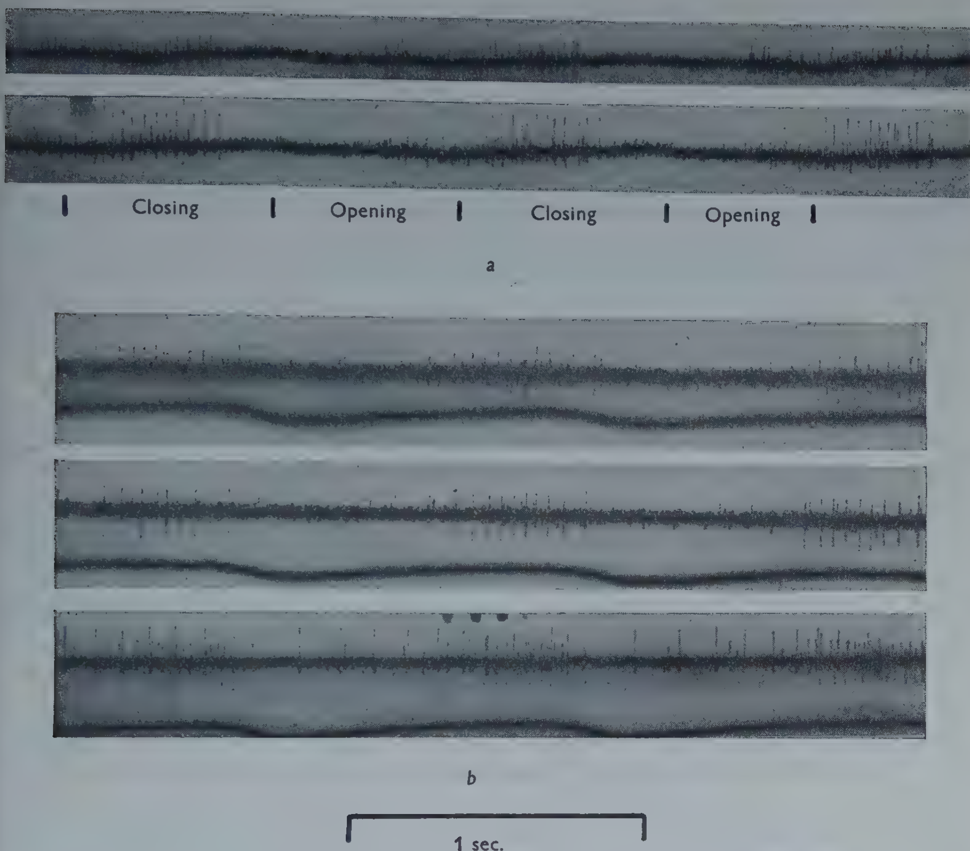

Fig. 3. Respiratory activity. The effect of small movements of the electrode on the discharge pattern. (*a*) Activity from the reticular formation near the VIIth motor nucleus. The second record was obtained from a point 0·05 mm. lower than the first. (*b*) Activity from the Vth motor nucleus. The second and third records were obtained from points 0·03 and 0·08 mm. lower than the first (down on movement trace = operculum closing).

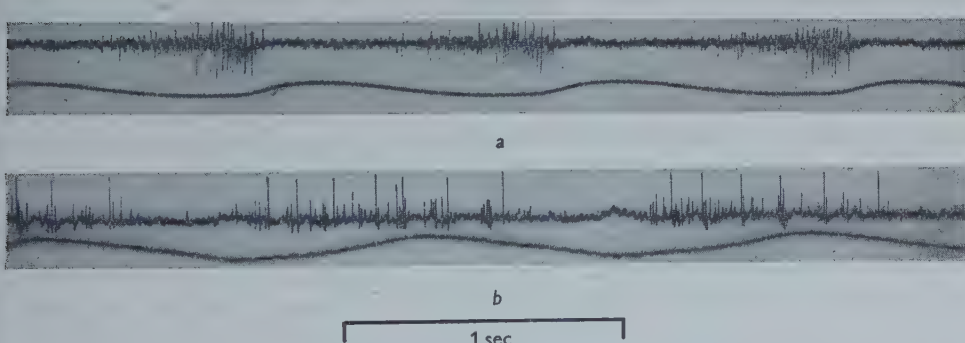

Fig. 4. Respiratory activity. Discharges showing phase, frequency and duration differences obtained from similar regions of the reticular formation near the anterior facial motor nucleus in different animals. Up on movement traces = operculum closing.

same animal. The two records of Fig. 4 were obtained from similar regions in the reticular formation of different animals and show duration, frequency and phase differences.

In the mammal the expiration and inspiration phases of the respiratory cycle are easily distinguished, and there is no difficulty in deciding whether activity in a respiratory neurone coincides with one or the other phase. The movements of the fish which correspond to the alternate expirations and inspirations of the mammal are the contraction and expansion of the whole pumping mechanism. A complete separation of these antagonistic phases of the respiratory cycle is not possible in the fish because the pump is a dual mechanism with the action of the buccal component slightly preceding that of the opercular one during normal activity (Hughes & Shelton, 1958). Consequently there are periods, which can occupy up to two-fifths of a complete respiratory cycle in the tench, when one part of the pumping apparatus is in one phase while the other part is in the opposite phase. Despite this complication it has been possible to make a fairly satisfactory distinction between opening and closing phase neurones in the majority of cases, and the regions in which these occurred have been plotted in Fig. 2 as squares and circles respectively. Inevitably there were a few phase-spanning discharges which were difficult to classify, and the two general types which occurred can be seen in Fig. 4. The large unit in Fig. 4*a* was active for a small proportion of a complete cycle and occurred at a time when opercular expansion and buccal contraction were going on simultaneously. The discharge in Fig. 4*b* was produced over a much larger proportion of the cycle so that it covered the change of phase (in this case from contraction to expansion) of both buccal and opercular components. These intermediate types of activity were classified in all cases with reference to the buccal component of the pumping mechanism. As Fig. 2 shows, a higher proportion of the respiratory neurones found were active during the closing phase. No segregation of neurones associated with the two antagonistic phases of the respiratory cycle could be detected.

DISCUSSION

The experiments described show that rhythmic bursts of action potential activity, at the same frequency as the breathing movements, can be detected by means of needle electrodes over a wide area of the tench medulla. In agreement with the conclusions of previous workers who have used electrodes with fairly large tip diameters (e.g. Salmoiraghi & Burns, 1960; Cohen & Wang, 1959), it seems likely that the activity was detected from nerve cell bodies and not from fibres. In many cases the electrode could be moved for distances of more than 50 μ (Fig. 3) without losing a particular unit completely. Activity coming from a nerve fibre would probably be much more sharply localized. Moreover, cell bodies could always be seen on histological examination of regions where activity was picked up. The area of the medulla occupied by the respiratory neurones corresponds with that which has been shown by transection experiments to be necessary for the co-ordination of normal breathing movements (Shelton, 1959). The rhythmic bursts of activity

came from cells situated beneath the IXth and Xth motor nuclei and in the neighbourhood of the VIIth motor nucleus. Some of the motor neurones forming the Vth and VIIth nuclei were also found to be rhythmically active, though this was to be expected as the main muscles of the pumping mechanism are innervated by the Vth and VIIth cranial nerves. These motor neurones forming the final common path to respiratory effectors, like the spinal motor neurones innervating the thoracic musculature of the mammal, may be regarded as being outside the integrating respiratory centre in the strict sense, but there seems to be little advantage in making such a distinction. Respiratory integration may go on right up to the level of the motor neurone; and schemes, such as most of those proposed for the mammal, which divide off the motor neurone and surrounding regions from the rest of the integrating apparatus, may be over-simplifications (Liljestrand, 1953).

The failure to detect respiratory neurones with many of the electrode insertions made in the respiratory area suggests that the fish respiratory centre, like that of the mammal, consists of a system of neurones dispersed through the grey matter of the medulla. The absence of any dense nuclei of respiratory neurones has been confirmed by searching the areas around the sites of successful electrode insertions. When several insertions were made within a radius of about 300 μ of a successful site it was quite common to find no respiratory discharges at all, even though multi-unit discharges occurred at the active region. It seems likely from this that the neurones are not evenly scattered through the respiratory area, but are arranged in very small groups; in this way it is possible to reconcile the high degree of localization of the active sites with the multi-unit discharges usually obtained from them. There is also lack of uniformity in the respiratory neurone distribution in a more general sense. The experiments have shown there to be a higher density of active sites in the region beneath the posterior border of the cerebellum and the anterior border of the facial lobe than there is elsewhere in the respiratory area.

These proposals on the arrangement and distribution of the neurones, which together make up the respiratory centre, are the outcome of results obtained from experiments on a large number of fish. The points plotted in Figs. 1 and 2 represent active sites found in all these experiments and give no indication of any individual variation which may exist. Because of the low probability of finding active units in a general search, and the time-consuming nature of the searching process which prevented an examination of large areas of medulla in any one animal, no data on individual variations are available from the experiments. It is possible that the boundaries of the respiratory centre, and the arrangement of the respiratory neurones within them, are the same in every individual and that the whole system is anatomically quite stable at all times. There is nothing in the results to suggest that this must be so; it is equally possible that the respiratory centre is labile and that variations in the distribution and density of active units occur in different animals, or in the same animal at different times. There is some evidence to support the view that the centre is not a rigidly fixed system of inter-connected neurones in a single individual. Von Baumgarten (1956) has shown that, in the mammal, the breathing of gas mixtures containing high tensions of oxygen causes an increase in

activity in existing expiratory neurones and the recruitment of many new neurones of this type. Eventually the expiratory neurones become dominant in the respiratory centre, the reverse of the normal situation when air is breathed. Similarly, Burns & Salmoiraghi (1960) have found that an increase in the tension of carbon dioxide in the respired air causes the recruitment of more rhythmically active units, in addition to increasing the discharge frequency in all the respiratory cells. It seems likely that the balance of respiratory neurones may be constantly changing by the recruitment of some units and the dropping out of others, as different conditions are encountered. The recording and transection experiments done so far on the fish centre have been under conditions of complete aeration of the water in which the animals were breathing. It would now be interesting to know what effects are produced in the general organization of the centre by changes in the gas tensions of the medium.

The way in which activity of a rhythmic nature is generated within the neurones of the respiratory centre is not clear. Adrian & Buytendijk (1931) suggested that the respiratory centre of the goldfish was capable of producing normal rhythmic activity when the brain was completely isolated. This proposal has since received the support of Hukuhara & Okada (1956) following their work on the isolated brain of the catfish and carp. The property of inherent rhythmicity, persisting in complete isolation, has been interpreted as evidence for the existence of a pacemaker within the centre. A pacemaker system is one in which rhythmic activity invades the whole centre from one or a few intrinsically rhythmic neurones. A great deal of the work on the mammalian centre has led many workers to reject the pacemaker hypothesis. The more generally accepted view is that the respiratory rhythm is generated by a widespread interaction of large numbers of neurones, none of which is intrinsically rhythmic in isolation (Wyss, 1954). The effects of local destruction of neurones within the tench centre suggest that here, too, the rhythm is not due to the action of a pacemaker, for in no case did the destruction of an active site stop rhythmic breathing. On the other hand, the transection experiments show that removal of the posterior part only of the proposed respiratory centre is effective in stopping the breathing movements. It is possible to reconcile this fact with the hypothesis of a rhythm due to reciprocal interaction between large numbers of neurones, if it is supposed that interaction breaks down when a given proportion of the population is removed. The evidence for either hypothesis is not compelling and a more intensive study is required of the inter-relationships of the neurones which have been described here as constituting the respiratory centre.

SUMMARY

1. The medulla of the tench brain was searched systematically by means of needle electrodes for rhythmic bursts of action potential activity coinciding with the breathing movements.

2. The neurones which produced these rhythmic bursts of activity were located in the grey matter, mainly beneath the IXth and Xth motor nuclei and in the region

round the VIIth motor nucleus. This type of activity was also found in some of the neurones forming the Vth and VIIth motor nuclei.

3. The respiratory neurones were not arranged in a discrete and homogenous nucleus anywhere in the medulla, but were scattered through the grey matter. The distribution was not uniform, the neurones tending to occur in very small groups. There was also a relatively higher density of respiratory neurones in the central, as compared with the more anterior and posterior, parts of the respiratory region. The possibility that variations may occur in the constitution of the respiratory centre, in different individuals and in the same individual at different times, is considered.

4. The manner in which neurones of the respiratory centre function to produce the rhythmic activity is discussed. Localized destruction of active respiratory regions, over a wide area of the medulla in different fish, was never followed by a breakdown in the rhythmic movements. This is interpreted as evidence against the existence of a pacemaker and favouring the hypothesis that the rhythm is produced by a general reciprocal interaction of large numbers of respiratory neurones.

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THE OXYGEN CAPACITY OF GOLDFISH (*CARASSIUS AURATUS* L.) BLOOD IN RELATION TO THERMAL ENVIRONMENT*

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(*Received* 26 *July* 1960)

INTRODUCTION

The exchange of respiratory gases between vertebrate animals and their environment always takes place through water and usually involves specialized structures such as lungs or gills. The transport of oxygen from the site at which it is acquired to the remaining parts of the body in which it is needed is the primary function of the blood. It is generally held that the presence of a respiratory pigment facilitates this function of the blood. The same pigment, haemoglobin, is found in the blood of all vertebrates, but, as Barcroft (1928) pointed out, no two haemoglobins appear to be the same.

When the conditions under which the respiratory exchange occurs are considered, it is evident that fish encounter greater variations in such important factors as hydrogen-ion concentration, oxygen concentration and temperature, than any air-breathing vertebrate. This is particularly true for freshwater species. Knowledge of the relationship between the biochemistry of haemoglobin and mammalian respiration raised questions regarding the adaptation of fish to the situations presented by their habits and habitats. Temperature, for instance, is known to have a marked effect upon the oxygen dissociation curve of mammalian blood (Krogh, 1941). By analogy, the oxygen transporting efficiency of fish blood would be expected to fall with decreasing temperature, yet many fish remain active over a fairly wide thermal range—particularly if the transition from one temperature to another is not too abrupt. Thus fish offer promising material with which to combine studies of ecology and physiology. A number of reviews deal with the literature relevant to the physiology of fish respiration; the more recent are by E. C. Black in Hoar, Black & Black (1951) and Fry in Brown (1957).

The general problem consists of relating the behaviour of the intact fish to the properties of its blood *in vitro*. The blood of fish exhibits a number of interspecific differences, including haemoglobin content (Hall & Gray, 1929), affinity for oxygen, and the extent to which the oxygen dissociation curve is displaced by carbon dioxide (Krogh & Leitch, 1919; Root, 1931; Willmer, 1934; Black, 1940).

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In addition to these biochemical differences, Hart (1943, 1944) showed that the amount of blood pumped by each beat of the heart varied with the species.

The term *Bohr effect* has been borrowed from mammalian physiology to describe the effect of carbon dioxide on the oxygen dissociation curve of the blood, although it is by no means certain that the mechanisms involved are the same. The greater sensitivity of fish blood in this respect prompted a number of studies of the effect of carbon dioxide on oxygen uptake by intact fish (Fry & Black, 1938; Irving, Black & Safford, 1939; Safford, 1940). These investigators sealed fish in bottles of water containing oxygen at atmospheric tension and varying amounts of carbon dioxide, left them to asphyxiate, and then measured residual oxygen and carbon dioxide tensions. Plotting these residual tensions of the two gases against one another, resulted in curves characteristic for a given species, and they appeared to bear the expected relationship to the sensitivity of the blood. The term *respiratory tolerance* has been used (Irving *et al.* 1939) to describe the ability of fish to remove dissolved oxygen from water in presence of carbon dioxide, but it could equally well apply to a tolerance of potassium cyanide. The specific term *carbon dioxide tolerance* is more descriptive of the phenomenon, and is used here.

The general relationship between these features of teleost respiration as revealed by previous investigations is shown in Table 1.

Table 1. *The general relationship between the habitats of fish and certain features of their respiratory physiology*

			Characteristics of the blood *in vitro*		
Ecological group	CO_2 tolerance	Stroke output of the heart	Hb content	Affinity for O_2	Sensitivity to CO_2
Cold-water species, e.g. trout	Low	Low	High	Low	High
Warm-water species, e.g. bullhead	High	High	Low	High	Low

Fry, Black & Black (1947) noted a seasonal change in the carbon dioxide tolerance curve of certain minnows, and reasoned that this change might be induced by differences in thermal environment. They tested this point by a series of asphyxiation experiments, using goldfish acclimatized to various temperatures over their thermal range, and found goldfish at higher temperatures to have greater carbon dioxide tolerance. Thus changes in thermal environment had induced, in the one species, differences previously considered to be interspecific.

The asphyxiation experiments do not indicate the manner in which thermal environment affects carbon dioxide tolerance, but Fry *et al.* (1947) suggested that their results might reflect changes in either the circulation of the blood or the chemistry of oxygen transportation.

It appeared that the blood of thermally acclimatized fish should be examined from at least three aspects: (1) oxygen capacity, (2) oxygen affinity, and (3) effects

of carbon dioxide upon the oxygen dissociation curve. This paper reports the results of measuring oxygen capacity of blood from goldfish acclimatized to temperatures near the extremes of their range.

MATERIAL AND METHODS

The oxygen capacity of blood is the amount of oxygen per unit volume with which it combines when fully saturated with that gas. Saturation is normally achieved with oxygen at its atmospheric pressure. Oxygen capacity is commonly expressed as volumes per cent (ml. O_2/100 ml. blood). The measurement may be made as O_2, Hb (haemoglobin) or Fe, and the following relationship between the three is generally accepted for mammalian blood:

Fe (mg. %)	Hb (g. %)	O_2 (vol. %)
1·00	0·298	0·40
3·36	1·000	1·34
2·50	0·744	1·00

The relationship shown above assumes that gasometric measurements are corrected for dissolved oxygen. For purposes of calculations in this paper, it is also assumed that this relationship is valid for fish blood, although direct clarification, which does not appear to have been made, is obviously desirable.

Experimental animals

Goldfish were chosen for these experiments because both their carbon dioxide and thermal tolerance, as well as their capacity for acclimatization, were known (Fry, Brett & Clawson, 1942; Fry, *et al.* 1947; Brett, 1946). They are also readily available and easily maintained.

A maximum of twelve to fifteen fish, averaging about 9 cm. in length, were kept in each of six aquaria measuring 12 × 12 × 20 in. and containing approximately 36 l. of water. The temperatures were controlled within ± 0·1° C. at 5, 10, 15, 20, 25 and 30° C.—or, as stated in the appropriate places, at temperatures near these levels. The constant temperature aquaria have been described by Brown (1951). Air bubbled vigorously through the water kept it thoroughly stirred and fully aerated.

The fish were fed a mixture of beef liver and baby food (Farex) prepared exactly as described by Farris (1950). Those at 5° C. were fed once per week; the remainder were fed every other day. The amount given was what they appeared to eat readily. Excess food and droppings were siphoned from the aquaria periodically.

Brett (1946) showed that acclimatization to thermal change is itself a function of temperature; i.e. acclimatization occurs more slowly at the cold end of the range than at the warm. His findings were borne in mind in acclimatizing fish used in these experiments. The fish were held at 10° C. for 2–3 weeks before transfer to the 5° C. aquarium, and no fish were used from that aquarium until they had been there a minimum of 3 weeks. The minimum acclimatization period for 15° C. and higher temperatures was 1 week.

Collection of blood samples

Blood from larger species of fish from local rivers and streams was used in working out analytical techniques. It was found that several analytical procedures applicable to mammalian blood were unsuitable for fish blood.

All fish were rendered unconscious before removal of blood. Larger fish were struck on the head, while for smaller ones one of two anaesthetics was used. Five per cent procaine hydrochloride was administered directly to the brain by hypodermic syringe after Kisch (1947). This rapidly produces unconsciousness without stopping the heart. Alternatively, 5 % urethane was administered by placing the fish in a bath until it lost consciousness. This method proved most satisfactory with goldfish. Blood was collected directly from the heart or ventral aorta. The pericardial cavity was exposed from the ventral side and the area dried with filter-paper. A paraffined needle attached to a small polyethylene tube was inserted, usually into the bulbus or ventral aorta, and the heart was allowed to pump out blood into a small polyethylene vessel under slight negative pressure applied by mouth. With very small fish the needle was inserted into the ventricle. Clotting was prevented by dusting the interior of the collecting apparatus with dry heparin. Goldfish at 5° C. often lacked sufficient cardiac vigour to pump blood into the tube. Where this occurred, the pericardial cavity was blotted dry, an incision was made in the heart, and the blood collected by suction as it leaked into the surrounding cavity, the area having been dusted with heparin.

Measurement of oxygen capacity

Attempts to measure the oxygen capacity of blood using the Van Slyke manometric apparatus and the method of Sendroy (1931) were disappointing because of lack of consistency in replicates. Following Prof. Roughton's suggestion, the carbon monoxide method was adopted, with minor modifications from Roughton & Root (1945). In what follows, the terms CO capacity and O_2 capacity are taken to be synonymous and used interchangeably.

Because of discrepancies between Van Slyke results and alkaline haematin analyses, to be described, it was thought advisable to check whether the van Slyke method could extract all the CO combined with the blood. This was not a question of time, since it had been ascertained that the 5 min. period was adequate for extraction to constant volume. It was a question of whether there was an unextractable fraction. The method was to reduce a sample of blood completely, then to add a known volume of CO, to measure the quantity of CO remaining after absorption by the blood, and finally to conduct a Van Slyke analysis. Results are shown in Fig. 1 A, and they indicate that the Van Slyke method as commonly used is entirely reliable. Hence it was taken as a standard to which other methods of analysis could be compared. The need for another method for measuring oxygen capacity arose from the fact that 0·1 ml. of blood was required for each measurement by the Van Slyke method, and this quantity was as much or more blood than could be obtained from each of the experimental fish. Preliminary experiments indicated

that oxygen capacity measurements were not comparable unless measurements of cellular content were also made concurrently. These considerations made it necessary to find a method whereby oxygen capacity could be determined using not more than 0·025 ml. of blood. Alternate methods that were tried included measurement of the iron content of the blood and several methods common to clinical haemoglobinometry.

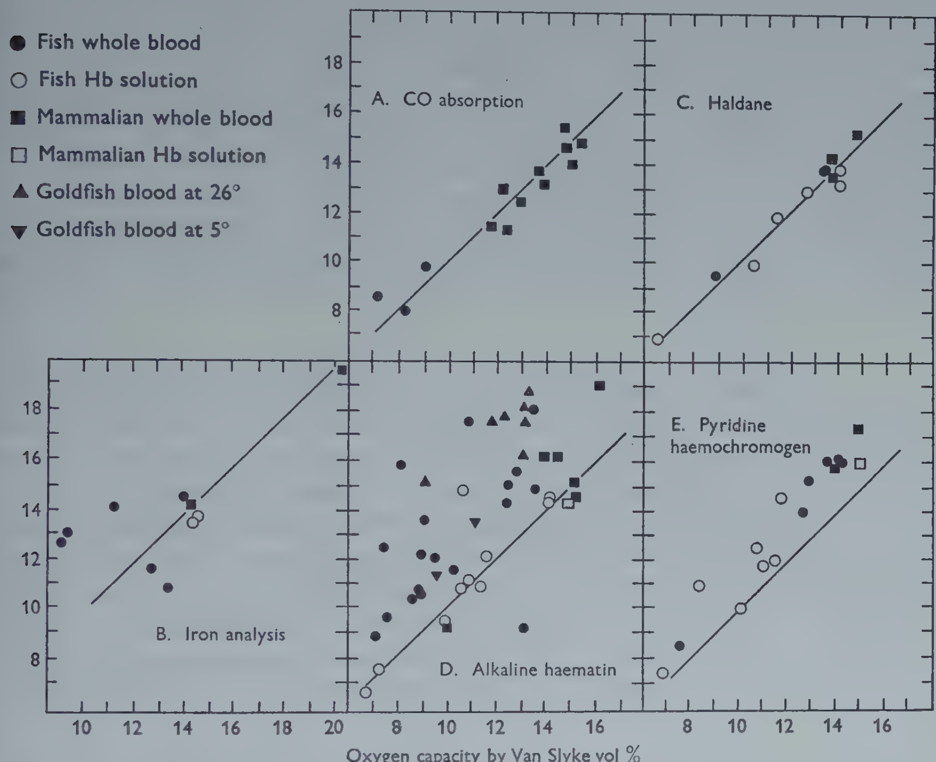

Fig. 1. Comparison of various methods of measuring O_2 capacity of blood. The standard method, gasometric determination of CO capacity with van Slyke apparatus, is checked against CO absorption in part A. In the remaining parts, various photometric methods of measuring Hb content are compared with the standard. Values are expressed as O_2 capacity in vol. %. Lines are drawn through points of equal capacity.

In dealing with mammalian blood, the measurement of iron content has frequently been taken as the standard to which other haemoglobinometric methods are compared, the oustanding instance being Harington (1952). Numerous methods have been prescribed for measuring iron in biological material and several were tried with goldfish blood. All of these methods begin with ashing the sample, either by incineration (dry ashing) or by means of powerful oxidizing agents such as HNO_3, H_2SO_4, $HClO_4$, H_2O_2, etc., used either singly or in various combinations (wet ashing). Ashing is followed by redissolving the iron, if necessary, and adjustment of pH and/or ionic state, depending upon the method by which the quantity of iron

is to be measured. Results presented in Fig. 1B were obtained by wet ashing followed by photometric measurement using an absorptiometer and either α-α′-dipyridyl (Hill, 1931) or catechol-3 (the disodium salt of 1,2-dihydroxybenzene-3,5-disulphonate) (Yoe & Jones, 1944) as an indicator. The latter indicator was favoured because it is specific for ferric ion, hence its use precluded reduction, a step that frequently resulted in cloudy solution.

The quantity of Hb in blood is a diagnostic observation in human medicine, and it has long been considered important for the practitioner to be able to make reasonably accurate measurements of the pigment. Numerous methods and related apparatus have been developed in response to this need. Photometric methods involving five Hb derivatives were tried in the course of the present work: alkaline haematin, carboxy-haemoglobin (Haldane), cyanhaematin, oxyhaemoglobin, and pyridine haemochromogen. All but the latter derivative were extensively tested by the Medical Research Council (MRC) (Harington, 1952). Their recommendations as to preparing the derivative and selecting a suitable filter for light absorption measurements was followed closely. A brief description of these methods follows:

Alkaline haematin method

0·023 ml. of blood was added to 4·975 ml. of N/10 NaOH with rapid stirring and the solution heated for 4 min. in boiling water. After cooling, the solution was transferred to a 1 cm. optical cell and read in an absorptiometer using Ilford Spectrum filter 605. For most of the determinations, an artificial standard (Gibson & Harrison, 1945) was used. Five ml. of this standard were heated along with the blood samples, then cooled and read in the same cells. A natural haematin standard was prepared by dissolving 38·0 mg. of haematin hydrochloride (B.D.H.) containing 8·46 % iron in about 100 ml. of borate buffer (19·07 g. $Na_2B_4O_7 . 10H_2O$ and 100 ml. of N/1 NaOH per litre), leaving it in the refrigerator to 'age' for 2 days, and then making it up to 500 ml. with the same buffer. Results with either standard agreed within 1 %.

Cyanhaematin method

0·025 ml. of blood was added to 2·5 ml. of N/10 HCl, stirred into solution, and allowed to stand for 15 min. 2·5 ml. of N/10 NaOH containing 2 % NaCN was added, and the solution was thoroughly mixed. It was then transferred to 1 cm. optical cell and read in an absorptiometer using Ilford Spectrum filter 605. Standard cyanhaematin solution was prepared by dissolving 14·6 mg. of crystalline haematin containing 8·46 % iron in 500 ml. of N/10 NaOH containing 1 % NaCN.

Haldane (COHb) method

0·025 ml. of blood was added to dilute ammonia (0·4 ml. conc. NH_4OH per litre) in a standard Haldane tube and the volume was immediately made up to the graduation with the dilute ammonia. The tube was then stoppered with a finger tip and the blood was shaken into solution. A minute amount of capryl alcohol was used to prevent foaming while pure CO was bubbled through the solution for 2 min.

The sample solutions were compared with a standard Haldane solution sealed in a matching tube. A special metal adaptor enabled these comparisons to be made in the absorptiometer using Ilford Spectrum filter 605.

Oxyhaemoglobin method

In some instances the blood samples were treated as in the Haldane method except that neither capryl alcohol nor CO were added. No permanent standard is available, but the Haldane standard was used for comparison and its equivalence derived by a correction factor in accordance with the MRC method.

Alternatively, 0·025 ml. of blood was added to 4·975 ml. of the dilute ammonia as used in the Haldane method and stirred rapidly into solution. The solution was then transferred to a 1 cm. optical cell and read in an absorptiometer with Ilford Spectrum filters 604 or 605. The concentration of Hb was then computed from the optical density by employing the MRC factors 0·330 and 0·498 as the respective optical densities with the two filters of blood containing 14·8 g. % Hb and diluted 1:200.

Pyridine haemochromogen method

The following method was based upon that of Collier (1944). 0·025 ml. of blood was added to 4·975 ml. of N/10 NaOH and the solution was heated and cooled as in the alkaline haematin method. 2 ml. of pyridine were added to the solution and, after thorough mixing, it was left to stand for 30 min. It was then reduced by the addition of about 5 mg. of $Na_2S_2O_4$, transferred to a 1 cm. optical cell, and read in the absorptiometer with Ilford Spectrum filter 604. 0·25 ml. of standard haematin solution was treated in the same manner. The standard solution was prepared by dissolving 66 mg. of crystalline haematin hydrochloride containing 8·46 % iron in 100 ml. of N/10 NaOH. Since the standard was 1 mM with respect to iron, it was theoretically equivalent to an oxygen capacity of 22·4 vol. %, but the total quantity of liquid in the final dilutions of blood and standard samples differed, being 7·000 for blood and 7·225 for the standard. Consequently, the standard was taken to be equivalent to an oxygen capacity of $22·4 \times 7·000/7·225 : 21·70$ vol. %.

In using both the iron method and the clinical methods, advantage was taken of a service of the MRC whereby subscribers may obtain periodically samples of blood of which the Hb content has been measured by the best modern techniques. These samples, presumably human blood, make up some of the points in Fig. 1. All photometric measurements were made with a Hilger 'Spekker' Absorptiometer.

Measurement of cellular content

The erythrocytes were enumerated in the standard manner using a Thoma diluting pipette and a haemocytometer slide. At first Hayem's diluting fluid was used, but subsequently that of George (1952) was found to be more satisfactory. The concentration of stain was reduced by a factor of four in order to get better results with fish blood.

Microhaematocrit tubes were used to determine the percentage of erythrocytes in the blood. They were centrifuged at 3000 rev./min. for 30 min. Duplicates usually agreed within 1 %.

It should be emphasized that, in dealing with the experimental fish, all three measurements—oxygen capacity, haemocytometer and haematocrit—were made upon aliquots from the same sample of blood. Hb solutions were prepared from fish blood by laking erythrocytes that had been washed 3 times in about 10 vol. of cold 0·9 % NaCl with gentle centrifugation. Laking was brought about either by alternate freezing and thawing in 10 vol. of the saline solution or by adding 1 vol. of cells to 10 vol. of distilled water while stirring rapidly. The cellular debris was then removed by centrifugation. Solutions of mammalian Hb prepared in this manner are said to be about 95 % pure.

From haemocytometer counts of the number of red blood cells (RBC), the percentage of RBC in the blood (haematocrit), and the oxygen capacity of the whole blood, the following values were derived as shown:

(1) Mean corpuscular volume:

$$\text{MCV} = \frac{\%\ \text{RBC} \times 10}{\text{Millions of RBC/mm.}^3} = \mu^3.$$

(2) Oxygen capacity of 100 ml. of erythrocytes:

$$\text{RBC} = \frac{\text{Whole blood capacity} \times 100}{\%\ \text{RBC}} = \text{vol. }\%.$$

(3) Oxygen capacity of single cells:

$$\frac{\text{Whole blood capacity} \times 10}{\text{Millions of RBC/mm.}^3} = \mu^3.$$

RESULTS

The results of tests to find a micro-method for measuring oxygen capacity are shown in Fig. 1. Fig. 1 B shows that iron analyses agree with Van Slyke measurements upon mammalian blood and fish Hb solutions, but exhibit considerable scatter when whole fish blood is used. While the results are too few to be conclusive, they draw attention to the fact that difficulties attending the use of iron content as a basis for haemoglobinometry are not limited to analytical techniques, but extend to interpretation of results. The scatter may have been due to varying amounts of non-Hb iron. Part of this non-Hb iron is in the plasma, but, as Lemberg & Legge (1949) point out, a larger portion is contained within the erythrocytes. The observation of Catton (1951) that there is a greater amount of debris in films of fish blood prepared for histological examination than in corresponding preparations of mammalian blood, has been confirmed in the present work. There appeared to be more 'ghosts' and general debris in haemocytometer preparations from fish blood than from human or sheep blood, and this was so even where clarity of plasma in haematocrits showed that no haemolysis had occured. If the debris results from

erythrocytes breaking down in the blood stream, then presumably the Hb becomes denatured, and the scatter observed in results of iron analysis may reflect Catton's morphological observation. In any event, the measurement of iron content of fish blood was not considered to be a suitable alternative to the Van Slyke method of measuring oxygen capacity.

The use of the alkaline haematin method for determining Hb in mammalian blood is fairly well established from reports of the MRC and from the work of Gibson & Harrison (1945), who prepared an artificial standard that is commercially available. The results of the present work are shown in Fig. 1 D and they indicate that while the method is satisfactory for mammalian blood and fish Hb solutions, with fish blood it gives very erratic results that tend to be much higher than Van Slyke.

There have been conflicting reports of the suitability of the alkaline haematin method to other than mammalian blood. Bankowski (1942), for instance, concluded that it was not satisfactory for avian blood, which resembles fish blood in having nucleated erythrocytes. From the present results it is thought to be unsuitable for fish blood.

The cyanhaematin method proved unsuitable because of difficulty with the first step, the conversion of Hb in the blood to acid haematin. Fish blood mixes more readily with acid than with alkaline solution, but the preparations frequently become cloudy. This is scarcely surprising inasmuch as Hb denatured by acid does not form a true solution but a colloidal suspension (Lemberg & Legge, 1949). The acid haematin method has been widely used in clinical medicine, where it is associated with the name of Sahli (Wintrobe, 1942), and has frequently been applied to non-mammalian blood, e.g. Schlicher (1926), Dombrowski (1953) and Black (1955). Admirable ingenuity has been displayed by some workers attempting to avoid or overcome the difficulties of applying the acid haematin method (Dukes & Schwarte, 1930; Elvehjem, 1931), but it appears simpler and more advisable to abandon the method.

The most obvious derivative to use is oxyhaemoglobin, but its use in an absorptiometer suffers from lack of a permanent standard. The MRC have attempted to overcome this lack by publishing what amount to extinction coefficients for a number of filters used in conjunction with certain Hb derivatives in both visual and photoelectric photometers. While the use of extinction coefficients is well established in spectrophotometry where very narrow portions of the spectrum are used, it has not been widely practised in obsorptiometry where exact reproducibility of filter characteristics is regarded with some doubt. This method was tried both by using the MRC factors for Ilford filters 604 and 605 directly, with the solution in 1 cm. cuvettes, and also by using the Haldane tubes and correcting the value of the Haldane standard by means of the MRC factors. Both of these methods gave good results, nevertheless more confidence was felt in using a method for which a permanent standard was available.

Results of the Haldane method (Fig. 1 C) were found to agree consistently with the Van Slyke methods; indeed the two methods appear to be interchangeable.

The pyridine haemochromogen method is not as convenient as the others in the series, but was found to be quite simple in practice, and gave precise results as is shown in Fig. 1E. All results fell above the line of theoretical agreement with the Van Slyke method, but agreement is achieved by multiplying the pyridine haemochromogen values by 0·878. Theoretically the Haldane method should be better than the pyridine haemochromogen one for measuring oxygen capacity, since any so-called 'inactive' fractions of Hb present in blood would be converted to haemochromogen. It is of interest to note that the unidentified substances which interfered with the alkaline haematin method appeared to have no effect upon measurements of pyridine haemochromogen.

Table 2. *Measurements on blood of pike and roach*

These fish had not been acclimatized to any specific temperature, but had been taken from the river and kept in aquaria at temperatures ranging from 10 to 15° C. The column headings have the following meanings: size is recorded as length in cm., weight in g. RBC 10^6 is the haemocytometer count in millions of erythrocytes per mm.^{3} of blood. Crit is the haematocrit reading in % erythrocytes. MCV (μ^3) is mean corpuscular volume in cubic microns; its derivation is shown in the text. Oxygen capacities are recorded in % by volume for whole blood and erythrocytes (cells), and in cubic microns for single cells. Capacity of the blood was measured gasometrically as carbon monoxide capacity and each recorded value is a mean of two or more determinations.

					Oxygen capacities		
	Size (cm./g.)	RBC (10^6)	Crit (%)	MCV (μ^3)	Whole blood (%)	Cells (%)	Single cell (μ^3)
Pike	38/417	1·7	27·0	159	8·07	30·2	48
	36/384	1·8	27·3	152	8·91	32·6	50
	32/224	1·8	30·8	171	9·67	31·4	54
	38/374	1·7	28·8	156	8·99	33·5	53
	39/371	1·4	31·7	223	7·65	25·5	54
	35/411	1·7	30·3	180	9·55	30·1	57
	37/363	1·6	29·5	185	9·86	33·4	62
Mean	36/364	1·7	29·1	175	8·96	31·0	54
Roach	23/207	2·0	48·5	242	14·50	29·9	73
	25/274	2·1	56·5	269	14·90	26·4	71
	23/195	1·7	43·0	253	11·53	26·9	68
	26/241	1·9	36·0	189	12·45	34·6	65
Mean	24/229	1·9	46·0	238	13·35	29·5	69

Records of measurements on the larger species of fish are included in Table 2. They had not been acclimatized to any particular temperature, but are included to give an indication of the differences in oxygen capacity shown by different members of the same species, and to show that the variation is reduced, hence the results made more comparable, if the erythrocyte content of the blood is taken into account. Thus oxygen capacity measurements on pike whole blood have a standard deviation of ±16%. This deviation is reduced to about half if cellular content is considered; the corresponding measurements of the oxygen capacity of the erythrocytes have a standard deviation of ±9%.

Time did not permit measurements of the capacity of goldfish blood acclimatized to a complete series of temperatures. The observations were therefore limited to temperatures near each end of the range, in the hope of obtaining maximal effects.

Some difficulty was experienced in maintaining fish in a condition of good health at temperatures below 10° C. Some individuals in the cold, which appeared to be otherwise healthy, had blood that was abnormal in appearance. Only values obtained from blood of normal appearance from fish of normal appearance are quoted in Table 3.

Table 3. *Measurements on blood of thermally acclimatized goldfish*

The column headings have the same meanings as for Table 2. The capacity measurements were made upon whole blood by the Haldane (carboxyhaemoglobin) method using an absorptiometer and each value is a mean of two or more determinations.

	Size (cm./g.)	RBC (10^6)	Crit (%)	MVC (μ^3)	Oxygen capacities: Whole blood (%)	Cells (%)	Single cell (μ^3)
Goldfish acclimatized to 5° C.							
	8·0/ 8·3	2·1	39·3	184	11·20	28·6	52
	8·0/ 8·9	2·0	35·9	178	10·67	29·7	53
	8·8/12·1	1·7	32·5	188	8·92	27·5	52
	—	2·0	32·5	166	9·71	29·8	50
	—	2·2	31·0	144	10·60	34·2	48
Mean	8·2/9·1	2·0	34·2	172	10·22	30·0	51
Goldfish acclimatized to 6° C.							
	10·4/20·3	1·2	24·8	212	6·69	27·0	57
	9·0/ —	2·2	40·0	180	9·75	24·4	44
	11·0/26·5	2·7	45·5	171	15·54	34·1	58
	9·8/19·5	2·1	33·5	163	13·18	39·3	64
Mean	10·1/22·1	2·1	36·0	181	11·29	31·2	56
Goldfish acclimatized to 26° C.							
	10·8/25·0	2·1	41·0	193	13·20	32·2	62
	9·5/19·5	1·6	31·4	193	9·20	29·3	56
	9·5/17·5	1·6	36·7	233	13·11	35·7	84
	9·5/18·5	1·7	38·7	226	13·11	33·9	77
	8·7/15·5	1·7	41·0	240	12·31	30·0	72
	9·8/19·0	1·6	39·3	240	13·31	33·9	81
	9·3/17·1	1·7	34·1	205	11·78	34·5	71
Mean	9·6/19·0	1·7	37·5	219	12·29	32·9	72
Goldfish acclimatized to 30° C.							
	9·4/12·6	1·3	25·0	188	6·65	26·6	50
	9·6/15·5	1·8	33·0	185	9·36	28·3	52
	9·4/16·2	2·0	38·3	192	12·50	32·6	63
	9·8/14·2	1·8	29·5	165	9·96	33·8	56
	9·8/14·9	1·9	28·5	152	8·59	30·2	46
Mean	9·6/14·7	1·8	30·9	176	9·41	30·3	53

The peculiar features which appeared in blood of some of the fish at low temperatures included low counts and low haematocrit values. The blood also was sometimes 'fragile', that is, exhibited considerable spontaneous haemolysis, usually without clotting. Excessive amounts of cellular debris and white cells were observed in the haemocytometer preparations. The erythrocytes also appeared to have an unusual refractive index, for they were nearly invisible in the counting fluid.

The results of measuring the oxygen capacity of blood from thermally acclimatized goldfish are shown in Tables 3 and 4, the former showing the variation from fish to fish. Before discussing these results, consideration should be given to what might be expected by way of changes in oxygen capacity.

Changes in the Hb content of blood in response to the environment might come about either by a change in the number of erythrocytes or by a change in the Hb concentration of the individual cells. Lemberg & Legge (1949) point out that the mean corpuscular Hb concentration is remarkably constant even with different vertebrate species, and in spite of variations in size and number of erythrocytes. This observation suggests that the second possibility is unlikely to be the basis of the mechanism. Numerous observations have shown that an increase in altitude of residence brings about an increase in oxygen capacity of the blood of various mammals, and that this change is in the number of erythrocytes (Prosser, Bishop, Brown, John & Wulf, 1950). In the experiments upon mammals, the oxygen tension has been lowered: in the present work, oxygen tension remained constant at about 155 mm. Hg, while the environmental change was via temperature. When mammals move to higher altitudes, oxygen demand is constant in the face of diminishing tension, whereas with fish living at increasing temperatures the oxygen demand is increasing in face of constant tension. The result could conceivably be the same; a drop in tissue oxygen which presumably would stimulate an increase in the oxygen capacity of the blood. Whether or not the point of stimulation is reached depends for mammals upon the increase in altitude, and for fish it may depend upon the increase in temperature. With men the point is passed in something less than 15,000 ft. altitude (Reynafarje, Berlin & Lawrence, 1954); with fish we do not know whether they can reach a temperature which would have a corresponding stimulatory effect. It is possible that fish may be unable to acclimatize to a temperature at which oxygen at a tension of 150 mm. will not meet the demand. It might be predicted from these considerations that if a change in the oxygen capacity of fish blood did occur with increase in temperature, it would be in the direction of increased capacity.

When measurements upon thermally acclimatized goldfish are reduced to oxygen capacities per 100 ml. of cells, it is apparent that no marked change occurs in the cellular content of Hb over the range from 5° to 30° C. This might have been predicted from the observations of Lemberg & Legge (1949), although observations of the constancy in erythrocyte Hb concentration stem mainly from homoiotherms. Table 3 shows that there is a fairly wide spread in cell count and in whole blood capacity at either end of the thermal range, but it is also apparent that any differences that exist at the temperature extremes are small and that differences resulting from a change of a few degrees in environmental temperature could not be discerned in these results. Hence all of the results have been placed in two groups, 'Warm' and 'Cold', in Table 4.

Calculations based upon Table 4 show that there is a significantly higher red cell count in the cold group of goldfish. This is not reflected in the whole blood capacity, for while the number of erythrocytes has increased in the cold group, their average size is smaller, with the net result of no change in capacity. It is noteworthy that the changes in question are so small as to require statistical tests to establish their validity. It follows from what has been said that there must be some difference in the oxygen capacity, and hence Hb content, of the individual

Table 4. *Summary of measurements on blood of thermally acclimatized goldfish*

The data from Table 3 are grouped at 'cold' (5 and 6° C.) and 'warm' (26 and 30° C.) for comparative purposes. T-tests ($P = 0{\cdot}05$) show the values marked with an asterisk to be significantly different.

Group	No. of fish	RBC (10^6)	Crit (%)	MCV (μ^3)	Oxygen capacities: Whole blood (%)	Oxygen capacities: Cells (%)	Oxygen capacities: Single cell
Cold	9	2·0*	35·0	176*	10·70	30·5	53*
Warm	12	1·7*	34·7	201*	11·09	31·7	64*

erythrocytes. It might appear that this statement is at variance with the observation of Lemberg & Legge (1949) and the conclusion reached in considering the capacities per 100 ml. of cells in the current work, but such is not so. The absolute amount of Hb per cell (mean corpuscular Hb, usually expressed in microgrammes per cell) may vary—i.e. small cells contain less Hb—but the concentration of Hb within each cell (mean corpuscular Hb concentration, expressed as %) remains fairly constant.

It should be mentioned that measurements by the alkaline haematin method upon the blood of the same goldfish showed an increase in 'alkaline haematin' content with increase in environmental temperature. Difficulties with this method have already been discussed. It was concluded that, if a true increase has been shown, it is an increase in non-haemoglobin substance.

SUMMARY

1. The Roughton & Root (1945) modification of the Van Slyke method for measuring haemoglobin content of blood as carboxyhaemoglobin is satisfactory for fish blood, provided at least 0·1 ml. is available.

2. Measurements of iron content are of doubtful value in estimating the oxygen capacity of fish blood.

3. The following photometric methods of determining haemoglobin content were found to be unreliable with fish blood: acid haematin, alkaline haematin and cyanhaematin

4. The following photometric procedures gave satisfactory measurements of the haemoglobin content of fish blood: Haldane (carboxyhaemoglobin), oxyhaemoglobin and pyridine haemochromogen.

5. Blood from goldfish acclimatized to temperatures near the extremes of their thermal range shows no difference from normal blood in oxygen capacity.

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SURVIVAL OF GOLDFISH IN PRESENCE OF CARBON MONOXIDE*

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INTRODUCTION

The introduction to a previous paper outlined the manner in which an increasing understanding of mammalian respiration stimulated an interest in the respiratory adaptations of fish. Of the various attempts to relate the biochemical properties of fish blood to behaviour of the intact animal, asphyxiation experiments deserve particular attention. Bohr, Hasselbalch & Krogh (1904) first called attention to the depression in the oxygen affinity of mammalian blood that is caused by carbon dioxide. The sensitivity of fish blood to carbon dioxide varies with the species, but in general it is much more sensitive than mammalian blood. Likewise the sensitivity of the intact fish to carbon dioxide has been shown to vary with the species, and more recently it has been suggested that this sensitivity may depend upon the temperature to which the fish is acclimatized (Fry, Black & Black, 1947). It is this sensitivity of the intact fish to carbon dioxide that is measured by the asphyxiation experiments mentioned above. Initially at least, the experiments were attempts to observe Bohr effect *in vivo*. Fish sealed in jars of water containing oxygen at atmospheric level and varying amounts of carbon dioxide are left until a cessation of respiratory movement indicates that death has been brought about by asphyxiation. Immediately after death of the fish, the residual amounts of oxygen and carbon dioxide are measured. From a graph of the results, a picture is obtained of the ability of the fish to remove dissolved oxygen from water when various concentrations of carbon dioxide are present. This ability has been called *respiratory tolerance* (Irving, Black & Safford, 1939), but Anthony (1960) pointed out that *carbon dioxide tolerance* is a better term, since it does not imply any inference as to the mechanism by which death is brought about. The latter term is used in this paper.

The inverse relationship that has been revealed between Bohr effect and carbon dioxide tolerance (cf. table 1 in Anthony, 1960) has generally been interpreted as evidence that carbon dioxide exerts its asphyxial effect upon the intact fish via the haemoglobin within its blood. Obviously such an interpretation assumes a certain dependence of fish upon haemoglobin. In particular it suggests that asphyxial

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oxygen tensions are related to haemoglobin content of fish blood. Recent discoveries in Antarctica drew attention to facts already in the literature that make it doubtful that fish as a class make much use of this respiratory pigment.

Nicloux (1923) reported the survival of three common teleosts, without apparent detriment, for 4 hr. in water equilibrated with air containing 2 % carbon monoxide. In 1926, Schlicher described a yearling carp with no erythrocytes in its blood. Matthews (1931) mentioned the existence of 'bloodless' species of fish in the Antarctic Ocean, and Ruud (1954) confirmed their existence with a description of three species, one of which may attain a kilogramme in weight. Commenting on Ruud's report and drawing attention to the supporting literature, Fox (1954) concluded that haemoglobin is an emergency precaution for fish and that oxygen carried in solution in their plasma serves adequately for normal purposes. Anthony (1956) suggested that haemoglobin acts in the role of a supercharger in fish respiration, but such an analogy leads to incorrect implications and must be discarded.

The suggestion that haemoglobin exists in goldfish blood merely for emergencies raises a number of questions with respect to previous work. Is haemoglobin essential to the normal activities of goldfish over any of their thermal range? How much of their activity depends upon presence of haemoglobin in their blood? Does the ability of goldfish to remove oxygen from water in the asphyxiation experiments depend upon this respiratory pigment—i.e. is asphyxial oxygen tension related to haemoglobin content of the blood? Ruud's report and Fox's comment upon it were published while work reported in the preceding paper was in progress. It was immediately apparent that Nicloux's work pointed to a possible means of answering some of the foregoing questions. Consequently, a series of experiments was set up in which goldfish were subjected to carbon monoxide. The results are presented herewith.

MATERIALS AND METHODS

Survival of goldfish in presence of carbon monoxide

Fish

Goldfish averaging 9–10 cm. in length were used in the experiments. The reasons for choosing these animals, the manner in which they were cared for, and the procedure by which they were thermally acclimatized has been described in a preceding paper (Anthony, 1960).

The equilibrium constant

The value of M, the CoHb/O_2Hb equilibrium constant for goldfish blood was very kindly determined for me by Prof. F. J. W. Roughton, F.R.S.

Blood for this purpose was collected from the exposed heart by means of a paraffin-lined hypodermic needle attached to a small plastic vessel. Clotting was prevented by dusting the interior of both needle and vessel with dry heparin. The Hb solution was prepared by laking 1 vol. of whole blood in 10 vol. of distilled water and removing cellular debris by centrifugation. The solution was diluted with teleost Ringer (Young, 1933) to a colour density suitable for observation by

reversion spectroscopy when viewed through the small tubes of the spectro-tonometers. The final dilution was about 1:150.

10 ml. portions of the Hb solutions were taken up in each of two tonometers and measurements of the fully oxygenated spectra were recorded. The solutions were then equilibrated with measured quantities of O_2 and CO and the measurements repeated. Finally measurement of the fully carboxylated spectra were recorded. From these measurements, the span—i.e. distance in Angstroms between α-bands of COHb and O_2Hb—and the partition constant, M, were calculated.

Gas mixtures

The mixtures used in these experiments were prepared with the aid of capillary flow meters (CFM). They were calibrated by means of a bubble flow meter (Barr, 1934). This method of calibration proved to be simpler and more accurate than the water displacement method that is frequently used. The bubble flow meter was also useful for checking total gas flow and setting a required flow through an uncalibrated CFM. Plotting the displacement of the manometer fluid in one arm against flow of air in ml./min. produced a smooth curve gently inflected upward for one CFM, but the graphical relationship was linear for the other three. All four were calibrated with air. Corrections to be applied to the indicated flow when other gases were passed through a CFM were derived from the following formula

$$\frac{NA}{NB} = \frac{tA}{tB},$$

where N = viscosity; t = time for a given volume of gas to flow through the capillary for a given temperature and pressure differences; A and B are the two gases. The corrections are shown in Table 1.

Table 1. *Conversion factors for capillary flowmeters calibrated with air*

(Derivation described in text.)

Gas	To convert flow of air to flow of gas	To convert flow of gas to flow of air
Carbon dioxide	1·24	0·81
Carbon monoxide	1·06	0·94
Nitrogen	1·06	0·94
Oxygen	0·91	1·10

The paper by Bailey (1954) proved useful in setting up the gas flow systems, shown diagrammatically in Fig. 1. Driers were not used, but use was made of both the water columns (A) as compensators and the fine control valves (B) described by Bailey for regulating gas flow. The plaster of Paris chokes were found to be unnecessary. The compensator used with CO had to be modified slightly so that excess CO would be conducted out of the laboratory. In addition to the fine control valves, single-way glass taps (C) were used in each gas line. A three-way glass tap (D), inserted beyond the point where the gases mixed, permitted sampling the gas mixture with only momentary interruption of its flow. O_2, CO and N_2 were

obtained from cylinders of compressed gas, and use was also made of the laboratory compressed air supply. The desired mixture of gases was initially set by means of CFM (*E*); the gas mixture was then checked by analysis using a Scholander-Roughton syringe (Roughton & Scholander, 1943), and finally the concentrations of gas in the water containing the fish were measured by Van Slyke analysis.

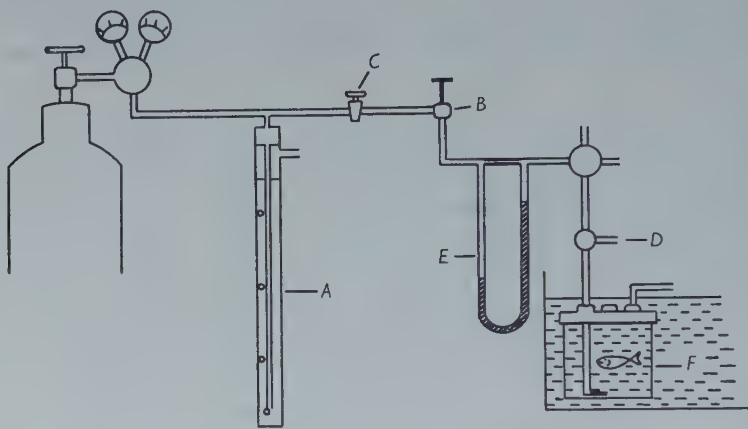

Fig. 1. Diagram of apparatus used in gas flow experiments. Explanation in text.

Flow experiments

Each fish was confined in about 400 ml. of water in jars of 500 ml. capacity. The jars (Fig. 1, *F*) were broad and flat and fitted with plastic screw caps. Glass tubes through which the gas mixtures passed into and out of the jars were inserted through rubber stoppers fitted into holes in the plastic cap. A third hole in the cap permitted sampling of water in the jar without interrupting the gas flow and was closed with a rubber stopper.

The tip of the glass inlet tube was drawn to a moderately small orifice and arranged horizontally near the bottom of the jar. The gas mixture issued as a small jet and exerted a slight stirring effect upon the water in the jar. The jars were filled with water from, and were suspended in, the aquarium containing the experimental fish. A broad rubber band around the outside junction of cap and jar produced a gas tight fit. The flow of air to control jars was adjusted by visual comparison to be approximately the same as for experimental jars.

Sampling the water

Straight, uncalibrated pipettes which would contain about 20 ml. were used in sampling the water. They were fashioned by adding the usual pipette tip to glass tubing about 1 cm. in diameter. The unconstricted upper end was fitted by means of a rubber stopper and a short glass tube to a length of rubber tubing and a glass mouthpiece. The tip of the pipette was fitted with a small piece of rubber tubing to enable it to be used in the Van Slyke cup in the usual manner. A small section of

glass tubing containing some glass wool was fitted over the tip when sampling. It prevented collection of excreta and other particulate material with the sample and also prevented contact of the tip with air during transfer of the sample from the aquarium to the Van Slyke analytical apparatus.

By holding these pipettes vertical and drawing in the water steadily, they could be filled readily without entrapping bubbles. Suction was applied either by mouth or by means of a 'Propipette'. A surplus of water was drawn up the rubber tubing to the mouthpiece. A clip on the tubing prevented loss of the sample.

The flow experiments were sampled through a special hole in the cap of the jar. Aeration of the contents of the jar was prevented by temporarily closing the exit so that gas was forced to pass out through the sampling hole. Where a series of samples was taken during the course of an experiment, the water level was maintained by addition of 20 ml. after each collection.

Where duplicate samples were taken, as in the asphyxiation experiments, one had to be stored 15–20 min. while the first was analysed. Otherwise, the samples were transferred within 2 min. to the chamber of the Van Slyke apparatus. Temporary storage was effected by submerging the pipette containing the sample, and with the filter over the tip, in the appropriate aquarium so that no change in temperature occurred from sampling to analysis.

Analysis of water samples

Analyses of water for dissolved gas content were carried out upon 10 ml. samples in the Van Slyke manometric gas analysis apparatus (Van Slyke & Neill, 1924; Peters & Van Slyke, 1932). A number of investigators have called attention to the desirability of manometric methods of analysing water for gas content where studies on aquatic respiration are concerned (Hall, 1923; Bosworth, O'Brien & Amberson, 1935; Zeuthen, 1947).

The Van Slyke chamber was de-aerated in the usual manner. The filter was removed from the tip of the sample pipette and the latter was then inserted beneath mercury in the cup and pressed lightly against the bottom. The clip was then removed from the rubber tubing so that the sample was free to flow. By raising momentarily the tip of the pipette, a few ml. of water were allowed to escape into the cup from the tip which had been briefly in contact with air. 10 ml. of the sample were then admitted to the chamber by lowering the mercury meniscus to the top of the numeral 10 above the 10 ml. graduation line. When the chamber was sealed it was found to contain exactly 10 ml., the difference having been made up from the quantity of sample contained between the top of the chamber and the mercury in the cup. The excess water in the sampling pipette served to protect from aeration the lower portion that was admitted to the chamber. I am indebted to Prof. Roughton for suggesting this simple method of transferring a measured aliquot of the water sample to the Van Slyke chamber.

The mercury was then lowered to the 50 ml. level and the gases were extracted by shaking for 5 min., a time that was found to be adequate for extraction to constant volume. CO_2 was absorbed by 1 ml. of 4% NaOH and O_2 by 1 ml. of alkaline

hydrosulphite solution containing sodium anthraquinone-2-sulphonate and prepared according to directions in Peters & Van Slyke (1932). Both of these solutions were thoroughly evacuated and stored under vacuum until immediately prior to use (Roughton & Root, 1945). The water was allowed to rise up and wash down the upper part of the chamber after each absorption and was then extracted at the 50 ml. level for about 30 sec. prior to making the subsequent pressure reading. Pressures were read with the water menisci at the 2 ml. level.

Following absorption of O_2, a bubble of the gas in the Van Slyke chamber was transferred to the syringe and its CO content was determined by absorption with Winkler's reagent (Roughton & Root, 1945). The remaining gas was expelled from the chamber and the pressure at the 2 ml. level was measured with only the liquid present. Factors for converting these pressure measurements to ml. of gas per litre of water were computed by the formula given in Peters & Van Slyke (1932).

The method was tested by analysing fully aerated distilled water and the results, as shown in Table 2, indicate that the method was quite precise and reasonably accurate.

Table 2. *Results of analyses by Van Slyke method of 10 ml. samples of distilled water equilibrated with air at a temperature of* 19·6° C.

(Concentration of dissolved gases in ml./l.)

	O_2	N_2	CO_2
Analyses	6·22	12·40	0·41
	6·27	12·35	0·35
	6·10	12·58	0·15
Mean	6·20	12·44	0·30
Expected	6·42	11·93	0·26
% error	3	4	13

Expressing gas content of water

The problem of expressing most meaningfully the gas content of water has been discussed by Fry in Brown (1957). He chose to express O_2 in mg./l. and CO_2 in partial pressure (mm. Hg). He was not concerned with expressing the concentration of other gases. Ricker (1934) pointed out that tables on the solubility of gases in water usually present those quantities dissolved from a 'dry' gas in contact with water at a pressure of 760 mm. Hg. Obviously this situation never obtains, and the tables are quite unpractical. A more practical table has been drawn up and presented as Table 3.

The results of analyses expressed in ml./l. were readily converted to a percentage of absolute saturation at a given temperature and hence the partial presure of the gas concerned was easily derived. The latter values for O_2 and CO were used in computing the percentage of COHb from the known value of M.

Table 3. *Solubility of gases in water when the total pressure of each gas plus aqueous tension is* 760 mm. Hg.

(Computed by multiplying the 'q' value for each gas by its molar fraction. Concentrations expressed in ml./l.)

Temp. (° C.)	Carbon dioxide	Carbon monoxide	Nitrogen	Oxygen
0	1705	35·2	23·8	49·0
5	1412	31·2	20·7	43·0
10	1190	27·8	18·4	37·9
15	1002	25·2	16·6	34·0
20	858	22·7	15·1	30·7
25	737	20·8	13·9	28·1
30	640	19·3	12·9	25·6

RESULTS

Preliminary experiments

The preliminary experiments were essentially a repetition of Nicloux's (1923) experiments, except that no attempt was made to analyse the blood of goldfish. Nicloux held fish for 4 hr. in a flask of water through which air containing 2 % CO was bubbled. The fish showed no signs of distress. He then removed the fish, obtained a sample of blood from a branchial artery, and analysed the blood for COHb. He found concentrations of COHb ranging from 86 to 92 %, but thought that some re-equilibration with air may have taken place in the 15 min. or so required for sampling.

Table 4. *Results of preliminary experiments with* CO *compared with those of Nicloux* (1923)

(Nicloux's fish weighed about 500 g.; goldfish about 15 g. All fish were alive at end of experiment. Nicloux analysed the blood for COHb. Bracketed values were calculated.)

Temp. (° C.)	CO (%)	Flow of air-CO mixture (ml./min.)	Time (hr.)	COHb (%)	Species
		Nicloux's experiments			
15	2·0	500	4·0	86	Carp
15	2·0	500	4·0	91	Pike
15	2·0	500	4·0	90	Eel
15	2·0	500	4·0	92	Eel
		Present experiments			
15	2·1	100	7·0	(87)	Goldfish
6	4·1	100	6·5	(92)	Goldfish
15	4·1	100	6·5	(92)	Goldfish
25	4·1	100	7·0	(92)	Goldfish
30	4·1	100	8·0	(92)	Goldfish

In these preliminary experiments, air containing 2 %, and later 4 % CO was used. The gas mixture was not passed through the jars until after admission of the fish. About 1 hr. was required for equilibration of gas and water at the prevailing rate of gas flow and that period is included in the times recorded in Table 4 for the preliminary experiments. The temperatures were 6, 15, 25 and 30° C. and in all of the

experiments, the fish were alive after exposure of 6–8 hr. Sometimes the treated fish appeared less active than the control, but after both had been liberated one could not be distinguished from the other in the aquarium. Thus it was concluded that goldfish over their entire thermal range can survive apparently unharmed for at least 8 hr. in aerated water containing 4% CO. No attempt was made to measure COHb concentration. It was expected to be near saturation. Subsequent determination of M permitted calculations of COHb concentrations shown in brackets in Table 4. They are lower than anticipated, and indicate that Nicloux's results probably were more accurate than he thought.

High concentrations of CO

In the next experiments goldfish were subjected to a mixture containing 80% CO and 20% O_2. This was the maximum concentration of CO that could be used without decreasing the tension of O_2 normally supplied by aeration. To increase the significance of measured survival times, the fish were introduced into solutions already in equilibrium with the gas mixture. The results are shown in Table 5. When the experiment at 5° C. was terminated, no difference in activity could be noted between control and experimental animal. At 15° C. the experimental animal, while quite lively, appeared to fatigue more readily upon handling. At 30° C. the experimental animal was alive and showed no ill effects after 24 hr., although it appeared more quiescent than the control. At 29 hr. it was dead.

Table 5. *Results of experiments exposing goldfish to high concentrations of* CO

(The analytical data are means of at least 6 determinations. The bracketed values are calculated.)

Gas mixtures				Water analyses in % absolute saturation					
T (°C)	Wt. (g.)	CO (%)	O_2 (%)	CO	O_2	N_2	CO_2	COHb (%)	Remarks
5	15	76·4	22·7	69·9	21·3	7·2	0·16	(99)	Alive after 48 hr.
15	19	80·0	20·0	71·2	18·7	11·5	0·14	(99)	Alive after 24 hr.
30	14	80·2	19·8	72·2	19·6	11·5	0·34	(99)	Alive after 24 hr., but dead after 29 hr.

The rapidity with which death from anoxia might be expected was tested by placing the fish in a jar of water equilibrated with N_2 that had been washed free of O_2 with alkaline hydrosulphite and rinsed with de-aerated water. Undoubtedly introduction of the fish resulted in some aeration of the water. The fish was distressed in less than 30 min., and dead in 2·25 hr. In a similar experiment the fish died in 1·5 hr. It is possible that traces of O_2 remained in the N_2 and prolonged the life of the fish; nevertheless, these tests indicate clearly that survival in presence of CO is not due to resistance to complete anoxia.

Asphyxiation experiments

The foregoing experiments indicate that goldfish supplied with oxygen at atmospheric tension can survive for some time in concentrations of CO calculated to convert practically all of their Hb to COHb. Presumably the plasma carries in solution enough oxygen to supply the tissues. In view of previous interpretations of asphyxiation experiments, it was now of interest to see how effectively a fish can reduce the oxygen content of water in presence of CO. With this in mind, asphyxiation experiments were set up with acclimatized goldfish, using techniques similar to those of Fry and his associates.

Flasks of water suspended in the experimental aquaria were equilibrated with a gas mixture containing O_2 at not less than atmospheric tension, CO estimated to block goldfish Hb, and CO_2 at as low a level as was practical. The jars containing the fish in the prepared solutions were submerged in the aquaria. The record of initial and final gas concentrations are results of duplicate analyses of water into which the fish was introduced and in which it later was found dead. Lack of gross respiratory movement was taken as a criterion of death. The diagnosis proved incorrect on two occasions, where fish subsequently recovered, but both had succeeded in reducing the O_2 content of the water to a low level. The results of these asphyxiation experiments are summarized in Table 6 and compared with similar data from experiments of Fry *et al.* (1947), in which CO was absent.

Table 6. *Summary of asphyxiation experiments*

(Left: results of present experiments using CO to saturate the Hb and keeping CO_2 to a minimum. Water was analysed for CO, CO_2 and O_2. Right: results quoted from experiments using no CO and with CO_2 at low tensions.)

Present experiments			Fry *et al.* (1947)	
Temp. (° C.)	Calculated COHb (%)	Asphyxial O_2 tension (mm.)	Temp. (° C.)	Asphyxial O_2 tension (mm.)
5	99	9	7	3·5
5	99	7	—	—
5	99	6	15	2·9
30	97	32	20	7·5
30	98	15	—	—
30	99	10	25	4·7
30	99	9	—	—
—	—	—	32	8·0
30	None	6	—	—

The asphyxial O_2 tensions in presence of CO tend to be slightly higher than those of Fry and his associates using low CO_2 tensions. This may be a true indication of the effect of CO, or a combined effect of CO and the CO_2 produced during the experiment. It may also be partially attributable to differences in the size of vessels, as suggested by Shepard (1955). In any event, the difference in asphyxial O_2 tensions in the two sets of experiments is not large, and the more general conclusion to be drawn from these results is that CO does not greatly impair the ability of

goldfish to remove oxygen from water in asphyxiation experiments. This is evidently so at either 5° or 30° C. and in presence of concentrations of CO calculated to convert at least 99 % of the Hb to COHb.

Table 7. *Relation between the equilibrium constant, M, of different haemoglobins and their span, which is the distance in Angstrom units between the α-bands of* O_2Hb *and* COHb.

(The value $M = \dfrac{\%\ COHb \times pO_2}{\%\ O_2Hb \times pCO_2}$ and $y = \dfrac{\log M}{\text{span}}$.

The values for goldfish haemoglobin are original and were determined by Prof. Roughton; other data expanded from Keilin and Wang (1946).)

Source of Hb	*M*	Span (A.)	*y*
Vertebrates			
Blood: (general range)	125–550	43–56	0·043–0·050
Man	300	54	0·046
Horse	550	56	0·043
Roach	210	52	0·045
Goldfish	63	63	0·029
Muscles (mammalian)	28–51	31–36	0·045–0·050
Invertebrates			
Gastrophilus larvae	0·67	95	−0·17
Leguminous plants (root nodules)	37	100	0·016

DISCUSSION

Keilin & Wang (1946) showed conclusively that the linear relationship between the span (y) and $\log M$ (K in their paper) does not hold good for all haemoglobins. Thus they brought to a close speculations initiated by Anson, Barcroft, Mirsky & Oinuma (1924) and continued by Barcroft (1928). The revival of this speculation upon the relationship between affinities and absorption maxima by Haurowitz & Hardin in Neurath & Bailey (1954) suggests that they may have overlooked the significance of the experiments by Keilin & Wang. It may be noted that the measurements made upon goldfish blood in the course of the present experiments also fail to support the suggested relationship and appear to be exceptional for vertebrates in this respect. Consequently it may be of interest to compare M, the COHb/O_2Hb partition coefficient, and y, the distance in Angstrom units between the α-bands of COHb and O_2Hb, for goldfish blood with values compiled by Keilin & Wang. This is done in Table 7. I have expanded the data of Keilin & Wang somewhat, but the source of the information is available in their paper. The present measurements were made for me by Prof. Roughton. Remarkably good agreement was shown by the two measurements from which the recorded value was derived, but it should be recalled that they were made upon one blood sample.

Results of experiments and cases of carbon monoxide poisoning in man have been summarized in tabular form by Pieters & Creyghton (1951). This summary indicates that a concentration of 1·28 % CO in air is fatally toxic to man within 3 minutes. Whether or not its effective time is ever quite so short, one cannot doubt the toxicity of CO to warm-blooded animals. Yet Haldane showed that this toxicity resided mainly in its ability to prevent Hb from transporting O_2. In one of his

experiments, a mouse survived unharmed in an atmosphere of CO containing two atmospheres of O_2 (Haldane & Priestley, 1935). However, these authors point out that CO may have other effects. Wells (1918) found that even low concentrations of CO were extremely toxic to five different species of fish, but his results suggest that the bullhead, *Ameiurus*, may survive for some time in fairly high concentrations. Survival of animals in presence of high concentrations of CO is rather surprising in view of the fact that it is known to combine with cytochrome oxidase. Keilin & Wang (1946) cite a value of 0·1 for the partition constant of that enzyme. From this it may be estimated that about 29 % of the enzyme would be blocked by the highest concentration of CO used in these experiments.

In discussing these experiments in which goldfish were exposed to various concentrations of CO, it will be assumed that the exposure resulted in the concentration of COHb shown by the calculation in the relevant tables. This assumption throws considerable stress upon the one measurement of M, but it does receive some support from the analytical data of Nicloux (1923). The stress is further relieved by the fact that the present value for M is lower than has been previously recorded for vertebrates; hence it might be expected that the calculated percentages of COHb are minimal values if they are in error.

Perhaps the most promising aspect of these experiments is that goldfish survived for fairly long periods of time in a concentration of CO much higher than is necessary to prevent their Hb from transporting O_2. They were able to do so over a fairly wide temperature range. In the one observation at 30° C., the time to death was over 24 hr.; the effective time at lower temperatures has not been observed, but it is greater than 48 hr. These observations indicate that CO may prove to be a useful tool in further examination of fish respiration. These tests have not been extended to other species of fish, nor to other cold-blooded vertebrates, but the results of such an extension should prove enlightening (cf. de Graaf, 1957).

Survival of goldfish exposed to CO in flow experiments indicates that standard (basal) activity in fully aerated water is independent of Hb over most of the thermal range—possibly even to 30° C—and that routine activity may have that independence over much of the lower range. By routine activity is meant that degree of natural activity such as one commonly observes in an aquarium (Fry; in Brown, 1957). The present experiments did not measure O_2 consumption, but activity was estimated from observation of control and experimental fish.

Despite concentrations of CO calculated to block all but a minute fraction of their Hb, goldfish were able to reduce the O_2 content of water to low levels in asphyxiation experiments. This was the most enlightening application of CO in the current experiments, for it indicates that the removal of O_2 under asphyxial conditions is not dependent upon the Hb content of goldfish blood. The reduction of the external O_2 supply can be accomplished by the circulating plasma. Thus the effect of CO_2 in the previous measurements of the CO_2 tolerance of this species was not limited to its effect upon the combination of O_2 with Hb. Hence no information regarding *in vivo* Bohr effect can be deduced from the CO_2 tolerance curves of this species. This observation may also apply to experiments measuring

the effects of CO_2 upon O_2 consumption of goldfish and it may well apply to other species.

In this respect, it is of interest to consider observations of Basu (1959) on the effect of CO_2 upon active O_2 consumption by various species of fish, including goldfish. His results clearly indicate that active O_2 consumption is influenced by CO_2 over the entire thermal range (cf. Basu, 1959, fig. 3E). He also confirmed the observation of Fry & Hart (1948) that active O_2 consumption by goldfish is independent of O_2 concentration down to a low level (cf. Basu, 1959, fig. 5C, 11). In view of this respiratory independence, as the latter aspect of their physiology has been called, it is interesting to find that O_2 consumption under active conditions is so immediately sensitive to CO_2. Unfortunately there does not seem to be any comparable measurement of the effect of CO_2 upon standard metabolism. It would be of great interest to see both standard and active O_2 consumption of goldfish measured in presence of sufficient CO to block their Hb. Although the extent to which other species of fish depend upon the Hb in their blood has not been tested, the results of Nicloux's (1923) experiments preclude the possibility that the present observations on goldfish are unique. Whereas goldfish with their remarkable degree of respiratory independence are also remarkably insensitive to CO, dependent species such as the trout, *Salvelinus fontinalis*, may prove to be quite sensitive. Fox (1954) recalls the observation of Hall (1930) that mackerel (*Scomber scombus*) must swim in order to obtain an adequate supply of O_2 and points out that this may indicate a constant dependence upon Hb that is probably unusual in fish.

Further reasons for doubting that CO_2 tolerance is an *in vivo* measure of Bohr effect may be found in the literature. The two curves reproduced in Fig. 2A are from the work of Fry *et al.* (1947). They are CO_2 tolerance curves for goldfish acclimatized to 7° and 32° C. Two features of these curves, the high tension at which CO_2 begins to show effect and the suddenness with which the effect occurs, are scarcely what would be expected of Bohr effect displayed *in vivo*, nor do those authors believe that the curves can be interpreted so simply (cf. Black *et al.* 1954). This point may be illustrated by reference to similar measurements upon the squid, *Loligo*. Fig. 3 is reproduced from a paper by Redfield & Goodkind (1929). Although the pigment in the blood of *Loligo* is not haemoglobin but haemocyanin, the CO_2 tolerance curve for this mollusc is probably a true picture of Bohr effect displayed by the intact animal. That this is likely is even more apparent from the nomographic analysis of squid blood prepared by these authors. Unfortunately there are not sufficient data with which to make a comparable analysis of goldfish blood.

It is also possible that the apparent effect of thermal environment upon the curves in Fig. 2A may not reflect a true difference in the sensitivity of the two groups of fish to high concentrations of CO_2, but rather a difference in solubility of that gas at the two temperatures. If quantity of CO_2 in mM./l. is plotted along the abscissa instead of partial pressure, all of the curves derived in the paper by Fry *et al.* (1947) inflect at about the same point, about 7 mM./l. (approx. 160 ml./l.), and the position of the two groups of fish indicated in Fig. 2A tend, if anything,

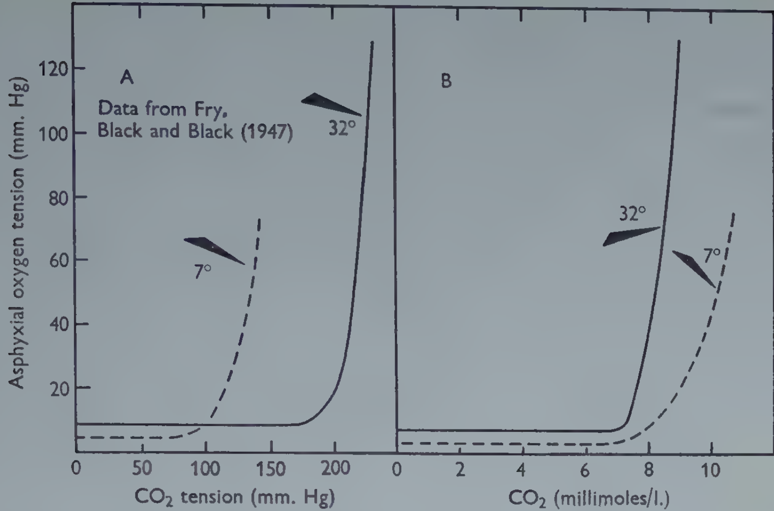

Fig. 2. Effects of CO_2 upon residual O_2 concentrations in asphyxiation experiments performed by Fry *et al.* (1947) with goldfish acclimatized to the temperatures shown. They plotted residual CO_2 concentrations as partial pressure as shown in A. The result of replotting their data with CO_2 concentration expressed as millimoles/litre is shown in B.

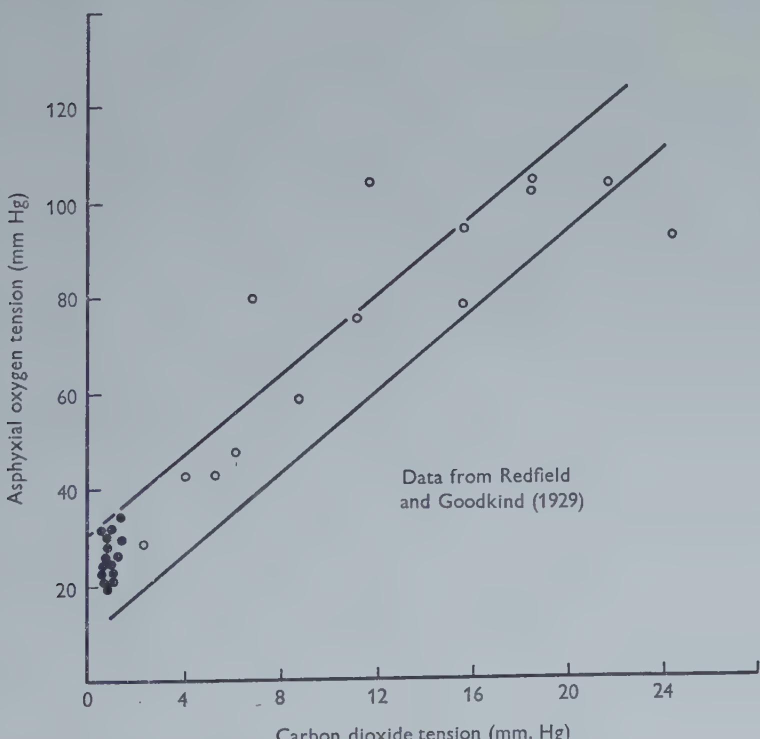

Fig. 3. Effects of CO_2 upon residual O_2 concentrations in asphyxiation experiments on the squid, which has haemocyanin as its respiratory pigment.

to be reversed. This point is illustrated in Fig. 2B. A similar criticism has been raised by Doudoroff (in Brown, 1957).

It can be calculated that a concentration of 7 mM. of CO_2/l., as mentioned above would result in a pH of about 4·3 if the water were unbuffered. The curves in Fig. 2A are reminiscent of the sharply inflected curves resulting from Powers (1932) observations on the effects of pH upon the ability of marine species to remove O_2 from water. The inflection in the latter curves occurred at a much lower hydrogen ion concentration—at pH 6·6–6·2. Powers used CO_2 to increase hydrogen concentration; hence his work fails to distinguish between effect of CO_2 *per se* and

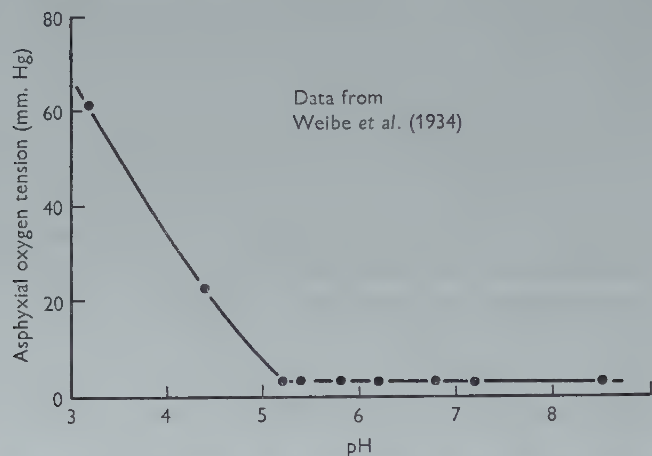

Fig. 4. The relation between hydrogen ion concentration and the level to which fish can remove dissolved oxygen in asphyxiation experiments.

effect of increased concentration of hydrogen ions. Weibe, McGavock, Fuller & Marcus (1934) used HCl to increase the hydrogen-ion concentration in an extension of the work of Powers and of Pruthi (1927). Their curves (Fig. 4) relating pH to concentration of O_2 at time of asphyxiation of goldfish are less sharply inflected than those of Powers for marine species and also less than the CO_2 tolerance curves in Fig. 2A. Nevertheless, it is noteworthy that the inflexion begins at about pH 5·2 in Fig. 4. This would suggest that in poorly buffered water the concentration of CO_2 at point of inflexion in Fig. 2A could inhibit O_2 uptake by virtue of hydrogen-ion concentration. On the other hand, it has been shown that CO_2 has a very marked effect upon certain intact animals even when it is applied in well-buffered solution (Jacobs, 1919), and Hall (1931) demonstrated effects of CO_2 upon the pufferfish, *Tetraodon*, that could not be ascribed to the associated hydrogen-ion concentration. Even the equivocal results of Powers (1932) suggest that it is CO_2 rather than the concomitant hydrogen-ion concentration that is inhibiting O_2 removal under asphyxial conditions, but it will require further experimental work to establish this point for goldfish.

Turning from the physical means by which CO_2 exerts its effect to the physiological route, it appears that our knowledge is equally, if not more, uncertain. At

least four activities are involved in removal of O_2 from water by fish. First of all, the water must be brought into intimate contact with the blood by being forced through the gills. This ventilation is achieved by respiratory movements. Secondly, the blood must take up the O_2 from the water. It is generally acknowledged that the transfer of respiratory gases between fish and their environment is by simple diffusion, but O_2 in the blood is held in both simple solution and chemical combination. Thirdly, circulation of the blood is essential to continuous removal of O_2 from water to tissues. Finally, the metabolic activities at cellular level must create a demand for O_2.

Since the plasma of goldfish has been shown to be an adequate vehicle for removing oxygen under asphyxial conditions, failure of the blood to take up oxygen is no longer a satisfactory explanation of the relationship between the asphyxial tensions of O_2 and CO_2 for this species. Hence attention is directed to ventilation, circulation, and metabolic activity as possible mediators of the effect of CO_2 upon asphyxial O_2 levels.

Present evidence indicates that CO_2 affects ventilation more immediately than it affects circulation. The literature contains few relevant accounts of circulation in fish (cf. Mott; in Brown, 1957). Most of the investigations have dealt with elasmobranchs; none has been concerned with the effects of CO_2 in high concentrations. Weibe *et al.* (1934) observed that fish may recover if transferred to well-aerated water after cessation of respiratory movements in asphyxiation experiments. Recovery of two goldfish in the present experiments confirms their observation and suggests in addition that cessation of respiratory movement precedes cessation of circulation. Furthermore, the criterion by which death was diagnosed in asphyxiation experiments favours the view that ventilation may be the activity by which asphyxial levels of O_2 and CO_2 are related. The well known narcotic effect of CO_2 suggests that its effect upon respiratory movement may be exerted via the central nervous system.

On the other hand, the toxic effect of CO_2 may arise from its action at cellular level. This possibility may be obscured by the means of diagnosing death in asphyxiation experiments and by the tacit assumption that O_2 continues to be consumed so long as movement can be discerned. It is not only possible but likely that movement continues for some time in the absence of O_2 consumption as measured by its disappearance from the water—i.e. the tissues go into O_2 debt. Present experiments with asphyxiation in N_2 indicate that a fairly large debt is accumulated. Hall (1931) found that increasing CO_2 tension reduced both O_2 consumption and frequency of respiratory movement of the pufferfish, *Tetraodon maculatus*. The reduction in frequency was accompanied by a marked increase in ventilation volume—i.e. volume of water passing through the gills per unit time—presumably through an increase in amplitude of respiratory movement. Hence there was, for a time at least, an increase in work during a decrease in O_2 consumption. This may be evidence that high concentrations of CO_2 block the mechanism by which the cellular batteries are normally recharged.

SUMMARY

1. The importance of Hb for certain aspects of goldfish respiration has been tested by converting it to COHb.

2. Goldfish exposed to 80% CO and 20% O_2 survived over 24 hr. at 30° C., and indefinitely longer at lower temperatures.

3. Routine activity of goldfish over most, if not all, of their thermal range does not depend upon Hb.

4. Removal of dissolved O_2 from water under asphyxial conditions does not depend upon Hb.

5. While the physiological basis of CO_2 tolerance curves remains unresolved, it can be said that they do not indicate an effect of CO_2 upon Hb.

6. Apparent changes in sensitivity to CO_2 that have been ascribed to changes in thermal environment are probably artefacts arising from variation in the solubility of CO_2.

During the course of this work, I enjoyed the inspiring supervision of Prof. F. J. W. Roughton, F.R.S. I am indebted to Prof. Sir James Gray, F.R.S., for accommodation in the Zoology Department and for his encouraging interest. The problem was called to my attention by Prof. F. R. Hayes, F.R.S.C., and I am further indebted to him for advice in preparing this paper.

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WEIGHT DISCRIMINATION BY *OCTOPUS*

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INTRODUCTION

Blinded octopuses can readily be taught to discriminate between objects touched provided that these differ in 'roughness'; objects with equally irregular surfaces, differing only in the distribution of irregularities, and objects of similar texture but different shape, are not distinguished. It seems that the animals are incapable of learning to recognize specific patterns of stimulation in the tactile sensory field, or to recognize how the arms must bend around objects grasped; they evidently distinguish things that they touch simply from the proportion of sense organs excited in the area of contact (Wells & Wells, 1957). The failure to take into account the relative positions of the sense organs excited seems best explained by supposing that *Octopus* is incapable of integrating proprioceptive with surface tactile information in learning to recognize objects by touch. This hypothesis is interesting because, if true, it would mean that the tactile learning system of *Octopus* is peculiarly simple, a mechanism limited to making only a quantitative distinction between surfaces touched. A direct test of the matter is to train octopuses to discriminate between objects of similar texture but different weight; such objects cannot be identified on a basis of surface tactile information alone, but can readily be distinguished by any animal capable of learning to recognize tension or pressure differences within the parts of its own body handling the objects.

The experiments reported here show that octopuses cannot be taught to discriminate between objects differing only in weight, although they very readily learn to make textural discriminations under the same conditions.

MATERIAL AND METHODS

Octopus vulgaris Lamarck of between 250 and 850 g. were used in these experiments. The animals were collected in the Bay of Naples and kept separately in large asbestos tanks. A few days before the beginning of training the octopuses were blinded by section of the optic nerves, as described in Wells & Wells (1956).

Blind octopuses can readily be trained to discriminate between the members of a pair of objects if they are rewarded (with a piece of fish) for taking one of the objects and passing it to the mouth, and punished (by means of a 5–10 V. a.c. shock, administered under water through electrodes attached to a probe) for taking the other; typical animals learn to make better than 75 % correct responses in a simple tactile discrimination within 20–30 trials, taking the 'positive' object whenever it is

presented, and rejecting the 'negative' by thrusting it away to arm's length. Errors at the start of an experiment are characteristically by failure to reject the negative object, since all unfamiliar objects are at first passed to the mouth.

For the present series of experiments two training routines were used. In one ('long-term' conditions) there were 8 trials per day at intervals of not less than 1 hr. systematized thus: + − + − + + − − first day, − + − + + − − + second day, and so on. In the second ('short-term' conditions) there were five times as many trials per day, arranged in two groups of twenty trials not less than 6 hr. apart. Within each group the trials, at intervals of 5 min., were systematized thus: + − + − + + − − + − + − + − − + + − + −. No gross differences in performance occurred under the two sets of conditions.

Table 1. *Details of the test objects used*

(All were cylindrical, 3·0 cm. long, cut from Perspex rod of 2·5 cm diameter.)

	Surface texture	Proportion of total surface cut away as grooves (%)	Weight in sea water to nearest g.
P1	Vertical grooves	30	5
P4	Smooth	—	5
P8	Circumferential grooves	50	5
P4H1	Smooth	—	45
P4H2	Smooth	—	15
P8H	Circumferential grooves	50	25

Pictures of P1, P4, P8, P4H1 and P8H are included in fig. 2.

Characteristics of the test objects used

Six different cylindrical Perspex objects were used in these experiments. Each object was 3·0 cm. long and 2·5 cm. in diameter. Three of the cylinders were left smooth (P4, P4H1 and P4H2), and three (P1, P8 and P8H) had deep, 1 mm. wide, grooves cut into their otherwise smooth surfaces. Two of the smooth objects (P4H1 and P4H2) and one of the grooved cylinders (P8H) were hollowed out, filled with lead and sealed with a Perspex plug. A more detailed specification of each object is given in Table 1, and pictures of most of them included in Fig. 2.

Behaviour of octopuses in handling the heavy objects

Blinded octopuses typically sit on the sides of their tanks, near to the water surface, with the arms outstretched. This makes it easy to present a test object by gently touching it against the back of one of the outstretched arms; the arm twists and the object is grasped by the suckers. In the experiments it was convenient to suspend each cylinder on a length of nylon line (attached to a knob at one end—see Fig. 2), by which the object could be recovered from the tank when the octopus had rejected or taken it and been rewarded or punished. Once the object was grasped by the suckers the line was left slack until the octopus had completed its reaction. In the case of the unweighted objects, weighing only 5 g. in sea water, slacking the line produced no appreciable postural changes to the octopus—the normal muscular

tone of the animal resting on the side of its tank was sufficient to support the weight. In the case of the weighted objects, and particularly of the very heavy P4H1 (45 g.), the situation was different. Here slacking the line frequently resulted in considerable passive extension of the arm grasping the object; the effect was transient, the animal rapidly contracting its arm and supporting the weight, but was nearly always clearly visible, so that an observer could identify the object presented by the octopus's reaction to it. It will be shown below that despite the obvious muscular adjustments needed to support the heavy cylinders, octopuses were unable to distinguish these from much lighter cylinders of similar surface texture.

EXPERIMENTAL RESULTS

Long-term experiments

Octopuses were trained to discriminate between P8 and P4, P1 and P4, and P1 and P8, all pairs of objects differing in texture, and between P4H2 and P4, which were alike in texture but different in weight. The animals readily learned to make the distinctions based on textural differences, but failed to learn to discriminate between P4, weighing 5 g., and P4H2, weighing three times as much (Fig. 1). In these experiments nearly all the errors were due to acceptance of the 'negative' object, which the animals were punished for taking, and in the weight discrimination tests five out of the six octopuses continued to take very nearly all of the objects presented throughout the 128 trials (16 days) of their training; these five animals together rejected only three objects in the whole course of training (2 negatives, 1 positive). The remaining animal concerned in the weight-discrimination experiment did reject a proportion of the test objects in the last 9 days of its training, but its performance (it rejected the negative object three times, and the positive eight times) was erratic, and gave no indication of learning to make the discrimination.

In the interpretation of these results it must be borne in mind that octopuses continuing to take both members of a pair of objects are not necessarily unable to discriminate between them; in retention tests, for instance, a reduction in the overriding tendency to react positively often reveals a capacity to discriminate that is otherwise masked (Wells & Wells, 1958). Although this was thought unlikely to account for the failure to discriminate between P4H2 and P4, further experiments were made under the short-term conditions, where the tendency to err only by acceptance of the negative object is normally less marked.

Short-term experiments

Four octopuses were trained to discriminate between P4 and P4H1 (a second smooth object, nine times as heavy as P4) at a rate of 40 trials per day. In Fig. 2 the performance of these animals is compared with that of 26 other octopuses trained to discriminate between the grooved P1 and the smooth P4. Once again the textural discrimination was learned rapidly, while the octopuses failed to make the weight discrimination. Over the period of training the proportion of positive responses by the four animals trained on P4H1/P4 declined until by the end of the

160 training trials less than half of the objects presented were accepted; under these conditions any tendency to discriminate should have been manifest were the octopuses at all capable of distinguishing between the objects. In fact the four animals together took the positive object 240 times and the negative 234 times in 320 presentations of each; even in the last 80 trials the scores were respectively 112 and 103 positive responses to the objects to be discriminated. Clearly these are chance scores and show no evidence of discrimination.

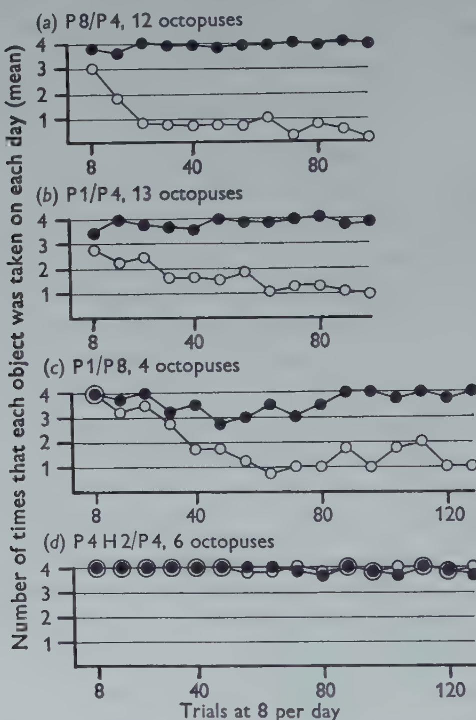

Fig. 1. A summary of training experiments carried out under the 'long-term' conditions. The plots show the number of times that each of the objects to be discriminated was taken on each day of 8 trials (4+, 4−); ● = positive object (which the octopus was rewarded for taking), ○ = negative object (octopus given a 6–10 V. a.c. shock for taking); where two points coincide the two are plotted ⊙. In (*a*), (*b*) and (*c*) octopuses were trained to discriminate between Perspex cylinders of the same weight differing in surface texture; the three discriminations are arranged in order of difficulty for *Octopus*. In (*d*) an attempt was made to train octopuses to distinguish between P4 and P4H2, an object three times as heavy as P4, but similarly smooth in texture. In each group approximately half of the animals were trained with one object as the positive and half with the other.

Two further experiments were made under the short-term conditions. Three animals were successfully trained to discriminate between P8H and P4, a discrimination for which both weight and texture cues were available, and an attempt was made to train two of these, together with three other (previously untrained) octopuses to distinguish between P8H and P8. The results of these experiments are plotted in Fig. 2.

The performance of the three animals trained to discriminate between P8H and P4 may be compared with that of the 26 animals trained to distinguish between P1 and P4 (Fig. 2). The two discriminations were learned about equally readily. Since P8H/P4 is on texture alone somewhat the easier discrimination of the two (see P8/P4 and P1/P4 in Fig. 1) this result again suggests that weight is not a property of objects that *Octopus* can learn to recognize.

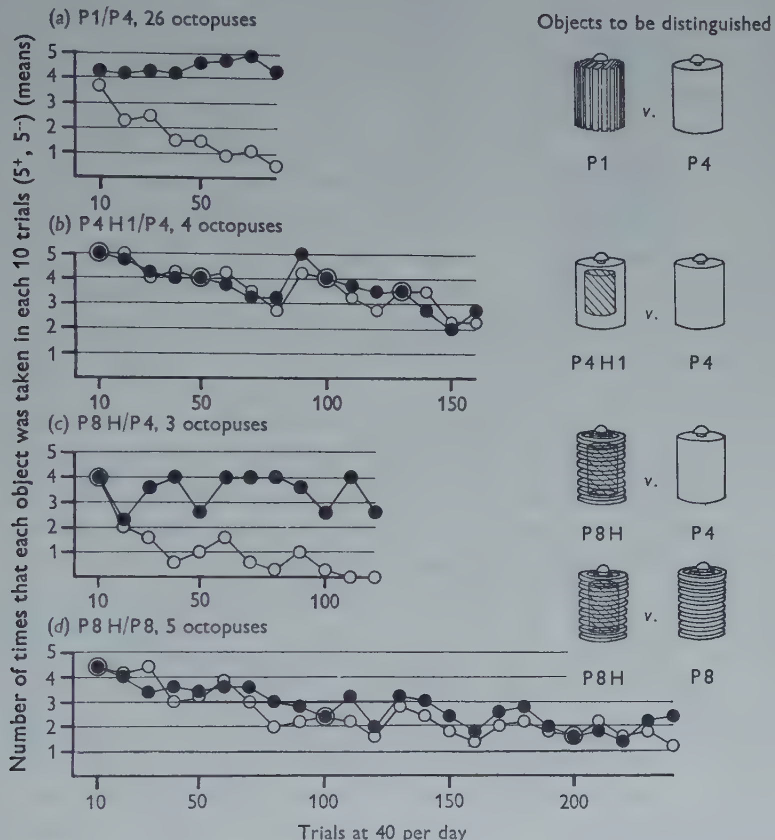

Fig. 2. The results of training experiments made under the 'short-term' conditions, with 40 trials per day in two groups of 20. In (*a*) twenty-six octopuses were trained to discriminate between the smooth P4 and the grooved P1, both objects being of the same weight (5 g.). In (*b*) an attempt was made to train four octopuses to distinguish between P4 and P4H1, a second smooth object nine times as heavy as the first. In (*c*) three animals were trained to discriminate between P8H and P4, which differed in both weight and texture. In (*d*) an attempt was made to train five animals, two of them already trained successfully under (*c*) to discriminate between P8H (weighing 25 g.) and P8 (5 g.). Experimental results plotted as in fig. 1. Some further details of individual scores are given in Table 2.

The attempt to train five octopuses to discriminate between P8H and P8 (P8H being five times as heavy as P8) was not successful (Fig. 2*d*). The performance of the animals was erratic, and although, over the 240 trials of training to which each was

Table 2. *Details of individual performances from the experiments summarized in Fig. 2*

(Columns A and B show scores in the first and second halves of the period of training)

		Number of times each object was taken				
		A		B		
Animal	Discrimination	+ve	−ve	+ve	−ve	Total trials
E14B	P4H1+/P4−	18	19	14	4	160
E21B	P4H1+/P4−	34	35	32	29	160
E23B	P4H1+/P4−	40	40	27	30	160
E38B	P4H1+/P4−	36	37	39	40	160
Totals		128	131	112	103	640
F2B	P8+/P8H−	49	46	57	50	240
F5B*	P8H+/P8−	41	34	31	26	240
F9B*	P8H+/P8−	21	23	24	16	240
F17B	P8H+/P8−	28	21	1	1	240
F23B	P8H+/P8−	58	59	23	20	240
Totals		197	183	136	113	1200

P4H1 was nine times the weight of P4, and P8H five times the weight of P8.
* F5B and F9B were pretrained to discriminate between P8H+ and P4 (see text).

subjected, every animal took the positive object slightly more often than the negative (regardless of which this was, P8H or P8), the difference in total number of takes was small; the positive object was taken 333 times out of a possible 600 and the negative 296 times ($\chi^2 = 1{\cdot}06$, $P = 0{\cdot}3$); details of individual scores are given in Table 2. When training was continued for a further 80 trials with three of the animals, the positive object was taken 91 times and the negative 65 times out of a possible 120 ($\chi^2 = 4{\cdot}7$, $P = 0{\cdot}03$). It is worth noting that this bias towards the positive object was found only in the P8H/P8 experiments, where there was a possibility of small textural differences between the objects due to inaccuracies in cutting the grooves; it has already been shown elsewhere that octopuses can detect quite minor differences of this sort (Wells & Wells, 1957). In the P4H1/P4 experiments, where the weight difference was greater than in P8H/P8, two out of the four animals took the negative object more often than the positive.

DISCUSSION

Strictly speaking, it is never possible to prove that an animal is unable to make a discrimination, since the failure may be the fault of the experimental conditions. When, however, animals regularly fail to make one sort of discrimination under conditions in which they readily learn others, there is strong evidence of a genuine lack of capacity. In the present instance the failure of octopuses to learn to discriminate between cylinders differing in weight under exactly the same conditions as those in which they rapidly and reliably learn to distinguish between similar cylinders differing in texture, can hardly mean otherwise than that *Octopus* is incapable of recognizing objects by their weight.

This is interesting because of its relation to facts that have already been collected about the organization of the central nervous system of *Octopus*. It has been shown that octopuses fail to make tactile discriminations that would depend upon their taking into account the relative positions of their tactile sense organs. They cannot, for example, be taught to discriminate between cylinders (of the P1/P8 type) that differ only in the pattern or orientation of grooves cut into them, and it seems that they cannot learn to discriminate between objects of different shape but similar texture (Wells & Wells, 1956, 1957). The failures are in discriminations that could in principle be made readily were the animals able *either* to scan the objects by moving their arms over them and integrate information about these movements, *or* to take into account the relative positions in space of the suckers in contact. Evidently octopuses cannot do either of these things in learning to recognize objects by touch, perhaps because there is no proprioceptive input to the inferior frontal system in the supraoesophageal part of the brain; for it is here that tactile learning takes place (Wells, 1959). The failure to learn to recognize the weight of objects supported by the arms is susceptible to the same explanation; proprioceptive information must enter the central nervous system—it is difficult to see how the animal could co-ordinate movements of the eight arms without it—but is evidently not available for integration with other sensory information in learned processes. A recent confirmation of this has come from the demonstration that learned visual discrimination of orientation in *Octopus* depends upon the constant orientation of the retina and not upon central integration of positional information with the visual input (Wells, 1960).

SUMMARY

1. Blinded octopuses can readily be trained to discriminate by touch between objects differing in texture.

2. They seem unable to discriminate between similar objects differing only in weight.

3. The relevance of this to what is already known about the use of proprioceptive information in learning by *Octopus* is discussed.

The author would like to thank the Director and staff of the Stazione zoologica di Napoli for their hospitality and for the facilities that they provided during the summers of 1958 and 1959, when these experiments were being made.

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STUDIES ON IONIC REGULATION IN *CARCINUS MAENAS* (L.)

I. SODIUM BALANCE

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INTRODUCTION

The fact that the body surface of the common shore crab, *Carcinus maenas*, is permeable to inorganic ions was established long ago. Bethe (1929, 1930) showed that changes in the external concentration of Ca, Mg and K were reflected in corresponding changes in the blood concentration of these ions. Following the demonstration by Schlieper (1929) that the body weight of *Carcinus* did not change when the animals were transferred to dilute sea water, Margaria (1931) correctly deduced that the body surface was permeable to the major blood ions (Na and Cl) from the fact that the total blood concentration decreased in a logarithmic manner when the animals were placed in dilute sea water.

The classical studies of Duval (1925) first showed that *Carcinus* could maintain its blood concentration well above that of the dilute sea water in which it was living. This was confirmed by Schlieper (1929) and subsequently by many other workers. For a number of years various explanations were put forward in an attempt to reconcile this fact with the conception of a freely permeable body surface. The correct explanation was suggested by Nagel (1934), who demonstrated that if animals adapted to a dilute medium were transferred to a medium of high concentration but still below that of the blood, the blood concentration was nevertheless increased. He postulated the inward secretion of salts from the medium to the blood, probably by cells of the branchial epithelium. Although, as Krogh (1939) pointed out, these experiments were not in themselves completely conclusive they provided strong evidence in favour of an active mechanism, which could transfer salts against a concentration gradient, participating in the maintenance of salt balance in these animals. This concept was finally put on a firm footing by the conclusive demonstration by Krogh (1937*a*, *b*, 1938) of the importance of such mechanisms in maintaining the relatively high blood concentrations found in many freshwater animals.

Finally, from a study of the concentration of individual ions in the blood of *Carcinus*, Webb (1940) showed that concentration differences were present even in animals from normal sea water which were practically iso-osmotic with the medium. He suggested that mechanisms concerned with the active absorption of certain ions

(Na, Cl, K and Ca) were operating even in these animals, and that they formed part of the over-all mechanism of ionic regulation, a process which underlay that of osmotic regulation.

In the process of osmotic regulation the quantitatively most important mechanisms are those responsible for the movement of sodium and chloride ions since these account for the bulk of the osmotic pressure exerted by the blood. The present study is concerned with a quantitative evaluation of those factors responsible for the maintenance of the blood sodium concentration in *Carcinus*.

Sodium balance in an animal results from the equality between the rates of sodium uptake and loss, each of which comprises the sum of the rates of sodium movement through a number of different channels. Thus sodium uptake is shared between a passive component due to diffusion through the permeable body surface and an active component represented by the active transport system. Sodium loss is partitioned between passive diffusion, sodium loss through the excretory organ and possibly also the active secretion of sodium. The level at which the blood sodium is set will depend on the values of these components, any of which may be variable. The fact that animals adapted to dilute sea water surpass those from normal sea water in their ability to maintain their blood sodium concentration above that of the medium may be due to the former having (*a*) reduced their surface permeability to sodium, (*b*) reduced sodium loss through the excretory organ, or (*c*) increased the rate of active sodium uptake. The use of the radioactive tracer technique allows these various possibilities to be investigated.

MATERIAL AND METHODS

The animals, supplied by the Dove Marine Laboratory, were collected from the Northumberland coast. They were subsequently kept in a laboratory aquarium supplied with circulating sea water. Over-all rates of sodium uptake and loss were determined by a tracer method using the sodium isotopes, ^{24}Na and ^{22}Na, and also by chemical measurements.

Sodium outflux was measured with ^{24}Na. The animals were loaded with the isotope by keeping them in 1 l. of sea water containing approximately 10 μc. ^{24}NaCl for 1 day. The animals were then transferred to a Perspex bath through which non-radioactive sea water could flow freely and were held firmly in position by means of a screw clamp across the breadth of the carapace. The radioactivity of the whole animal was measured by means of a Geiger counter placed immediately beneath the bath and the decrease in radioactivity as the tracer was washed out was recorded by means of a recording ratemeter. The apparatus is shown in Fig. 1. During a period of washing the animal's radioactivity decreased exponentially according to the relation $y = y_0 e^{-t/T}$, where y is the recorded activity at time t; y_0 is the initial activity and T is the time constant. Hence the outflux, M_{out}, could be calculated from the relation $M_{\text{out}} = A/T$, where A is the total freely exchangeable internal sodium.

Sodium influx was measured with ^{22}Na in an apparatus similar to that described

previously for influx determinations on *Astacus pallipes* (Shaw, 1960*a*). With this method it is essential that the internal sodium content should greatly exceed that in the external solution and therefore it could only be used for low external sodium concentrations. At higher concentrations the influx was calculated from chemical measurements of the net sodium loss rate using the relation: influx = outflux minus net sodium loss rate. The measured influx was made up of both active transport and passive diffusion components.

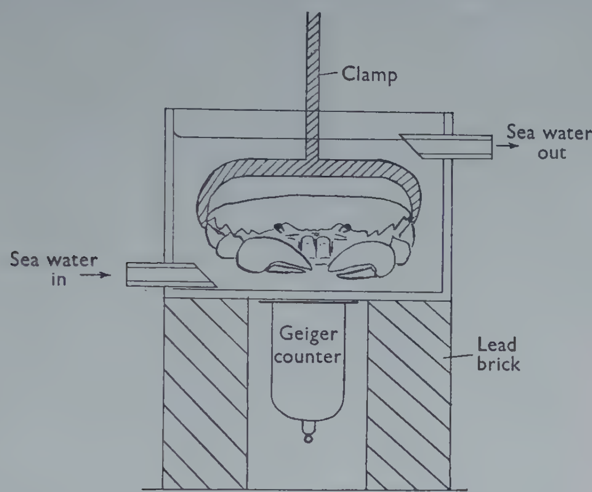

Fig. 1. The apparatus for the measurement of ^{24}Na outflux.

The rate of urine production was calculated from the rate of penetration of sulphate through the body surface and from measurements of the concentration of sulphate in the blood and urine. The calculation is described in a later section. Rate of penetration of sulphate and its relative concentration in blood and urine were measured by means of the sulphur isotope, ^{35}S, in the form of inorganic sulphate. The radioactivity of blood and urine samples was determined on diluted samples evaporated to dryness in a standard planchet and counted by means of a very thin-walled end-window Geiger counter.

Sodium concentrations were measured by means of an EEL flame photometer after suitable dilution of the sample.

RESULTS

(*a*) *Blood sodium concentration*

The blood sodium concentration was measured in animals adapted to a wide range of external concentrations from normal sea water to 20% sea water. The latter was the lowest concentration at which the animals would survive. The results are shown in Fig. 2, where a number of interesting features are displayed. The blood sodium concentration in normal sea water was generally slightly above that of the medium.

This agrees with the results of Webb (1940), although the concentration difference was never quite as great as he had found. As the external concentration decreased the blood concentration first fell and was then held relatively constant at about 375 mM./l. Na as the external concentration decreased by a further 100 mM./l. At external concentrations below 250 mM./l. the blood sodium concentration decreased steadily and the rate of fall was approximately proportional to that of the outside concentration. The significance of this behaviour is discussed in detail below after the result of the outflux and influx measurements have been presented. A similar behaviour with respect to the blood chloride has been previously described (Shaw, 1955*b*).

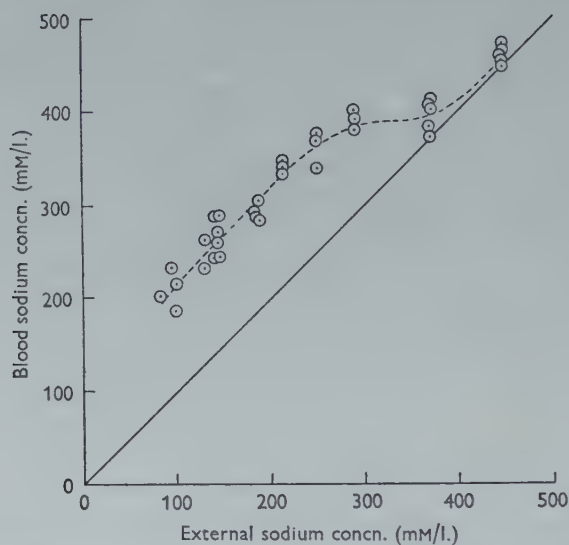

Fig. 2. The relation between the sodium concentration of the blood and that of the external solution.

(*b*) *Sodium outflux*

The first problem was to measure the rate of sodium loss in crabs from normal sea water. The ^{24}Na outflux in these animals was determined by the method described on p. 136 and the results of a number of the experiments are shown in Fig. 3. Here the logarithm of the measured radioactivity of the animal is related to the time after washing with non-active sea water had commenced. This relationship was linear in all cases so that the time constant for the loss of the isotope could be determined from the slope of the lines by the equation $\ln (y/y_0) = -t/T$ after correction for the decay of the isotope. Values of the time constant, T, are shown in Table 1. The mean value was 6·9 hr.

The outflux, M_{out}, was calculated from the relation, $M_{out} = A/T$, where A is the total internal sodium which freely exchanges with sodium in the medium. The internal sodium is largely confined to the blood and since the blood volume in *Carcinus* has been estimated at 35% of the body weight (Webb, 1940) it is possible to find the total blood sodium. Some sodium is, however, present in the tissues. The

muscle fibres contain 54 mM. sodium/kg. water (Shaw, 1955*a*) and this sodium exchanges freely with that in the blood (Shaw, 1958). If the tissue volume as a whole is similar to the blood volume and has the same sodium concentration as that found in the muscle fibres then the tissue sodium content is $35 \times (460/54) = 3\%$ of the body weight of a solution of the same sodium concentration as the blood (460 mM./l.). This gives the total internal sodium content as a volume of solution containing 460 mM./l. Na equivalent to 38% body weight (v/w). A comparable figure of

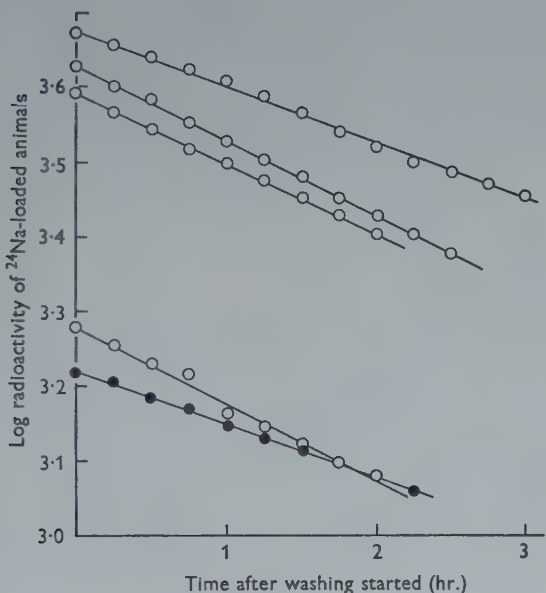

Fig. 3. The relation between the radioactivity of ^{24}Na-loaded animals and the time after washing with non-radioactive sea water.

Table 1. *The time constants for sodium outflux in animals from normal sea water*

Crab no.	Time constant (hr.)
1	7·5
2	7·5
3	9·0
4	8·0
7	6·5
9	6·0
10	7·4
11	5·8
12	6·8
13	4·4
14	8·6
15	5·0
Mean	6·9
S.D.	±1·4

37·1% was found in the crayfish (Shaw, 1959). For a 50 g. crab the total internal sodium would, therefore, be $50 \times 0{\cdot}38 \times 460 = 8740\,\mu$M. Na. Thus the outflux for animals from normal sea water is $8740/6{\cdot}9 = 1267\,\mu$M./hr. This is a rapid rate of exchange and represents a loss of 14·5% of internal sodium per hour.

It may be argued that tracer outflux measurements do not give a true measure of the rate of sodium loss because of the possible presence of an exchange diffusion component in the outflux. This would lead to an overestimation of the loss rate. The validity of the tracer method was therefore checked by chemical measurements on the rate of fall of blood sodium concentration when animals were transferred to dilute sea water. Small blood samples were withdrawn from the animals at intervals and the sodium concentration was measured. This was continued until a constant blood concentration was reached. If sodium was diffusing out through the body surface then the blood sodium concentration, C, should follow the relation:

$$C = (C_I - C_F)e^{-t/T} + C_F,$$

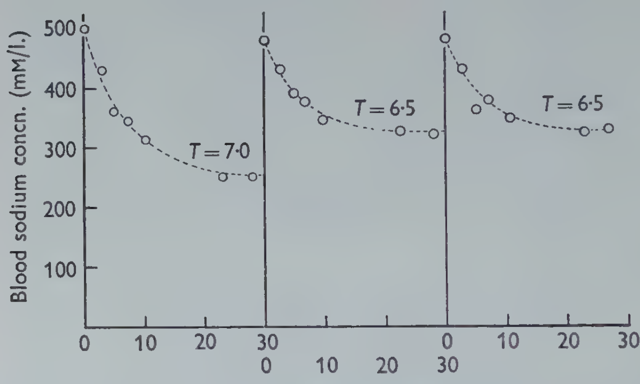

Fig. 4. The decrease in blood sodium concentration in animals transferred from normal to dilute sea water.

where C_I is the initial and C_F the final blood sodium concentrations. The experimental results are shown in Fig. 4, together with curves fitting these results and obeying the above relation. In the three animals values of $T = 6{\cdot}5$, $6{\cdot}5$ and $7{\cdot}0$ hr. gave a good fit and were in excellent agreement with the values of T determined by the tracer method. Therefore there is good reason to believe that the latter method gives a true measure of the rate of sodium loss. It also may be noted that Margaria (1931), who measured the rate of decline of the total blood concentration in *Carcinus* when they were transferred to dilute sea water, obtained results which were almost identical with the present ones for sodium.

If the ability of the animals to maintain their blood sodium concentration in dilute sea water is associated with a reduction in the surface permeability or a reduction in the loss of sodium through the excretory organ then this should be revealed by a comparison of the outflux time-constants of animals adapted to normal and to dilute sea water. Measurements were therefore made on animals from

normal sea water as before; the animals were then adapted to 50% sea water and the measurements were repeated. The results are shown in Table 2. There was no evidence of an increase in the time constant so that a reduction in the permeability of the surface membrane cannot play a significant part in the regulatory process. In most cases the time constant was actually decreased and this can almost certainly be explained by the increased rate of urine production found under these circumstances (see next section).

Table 2. *The time constants for sodium outflux in animals from normal and 50% sea water*

	Time constant (hr.)	
Crab no.	Normal sea water	50% sea water
16	6·5	6·5
17	6·0	6·2
18	7·4	6·8
19	6·8	5·6
20	8·6	6·8
Mean	7·1	6·4

(*c*) *Rate of urine production*

Nagel (1934) showed that *Carcinus* from normal and from dilute sea water produced an excretory fluid which was similar in composition to the blood both with respect to the total concentration and to the concentration of chloride. It therefore follows that if urine production plays a role in the regulatory mechanism then in dilute sea water the rate of production must be less than that in normal sea water. Previous measurements make this unlikely. Thus Nagel (1934) found the rate of flow in normal sea water was 10% body weight per day and this was increased in 50% sea water to 17% body weight per day. Bethe, von Holst & Huf (1935) found a rate of 7·2% body weight per day for animals from 65% sea water. Webb (1940) argues that the rate found by Nagel (1934) for normal animals was too high and, himself, obtained a much lower figure of 4·7%. In view of these discrepancies it is not possible to be certain that the loss through the urine is not reduced in dilute sea water.

All the previous measurements of urine flow have been made by measuring the increase in body weight after the excretory pores have been blocked by dental cement. This method is open to some obvious objections particularly at high rates of flow and it was therefore decided to attempt to measure the rate by another method in order to obtain independent confirmation of the figures, together with extended measurements of the rate of flow in dilute sea water.

It is possible to deduce the rate of urine production from the concentration of a substance in the urine providing this substance is entering the blood at a constant and known rate. It is possible, for example, to inject a substance like inulin at a constant rate into the blood of some animals and measure the concentration of inulin in the urine. *Carcinus* has the unusual advantage that in its normal environ-

ment such a situation is already at hand. The concentration of sulphate in the blood is maintained below that of the external medium by the activity of the excretory organ, and it will be assumed that the excretory organ is the sole agency whereby this difference in concentration is maintained. If the permeability of the body surface to sulphate is known it is only necessary to measure the concentration of sulphate in the urine and in the blood relative to that in the medium for a calculation of urine flow to be made.

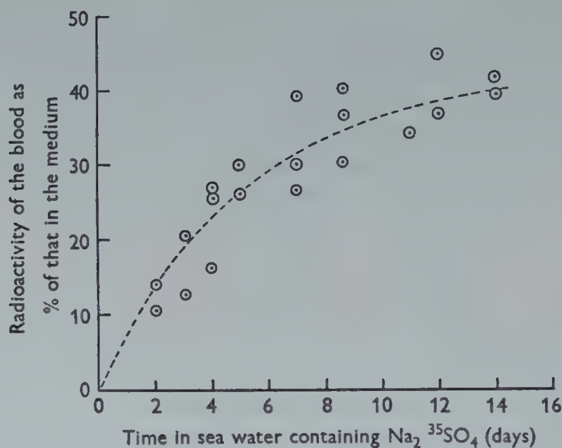

Fig. 5. The rate of penetration of inorganic sulphate from the external solution into the blood, measured with ^{35}S-labelled sulphate.

The first step was to measure the rate of penetration of inorganic sulphate. This was accomplished by keeping an animal in 400 ml. of normal sea water containing 50 μc. of ^{35}S in the form of inorganic sulphate. Blood samples were withdrawn at intervals and the radioactivity of the sample was compared with that of a sample of the sea water of equal volume. The results from several animals were pooled and are shown in Fig. 5. The points were rather scattered but can be approximately fitted by the relation, $A = A_F\,(1-e^{-t/T})$, where A is the radioactivity of the blood as a percentage of the activity of the medium, A_F is the final percentage activity of the blood and T is the time constant, equal to 5 days. It may be noted that the rate of penetration may vary from one animal to another, and that by using an average value for the time constant in the calculation of the urine flow the value so obtained has no significance for a given individual but only for the group mean.

To make the flow-rate measurements a number of animals were placed individually in dishes containing sea water with added tracer and left for at least 14 days to achieve a steady state. Samples of blood and urine were then taken and their radioactivities were compared with that of a sample of the sea water. The final calculation was made as follows. Let V_b = the blood volume, W the body weight, V_u the urine volume per day, T the time constant for sulphate penetration, M the concentration of sulphate in the medium, B_p the blood sulphate concentration as a percentage of the concentration in the medium, and U_p the urine sulphate con-

centration as a percentage of the concentration in the medium. Now sulphate influx is MV_b/T.

Assuming that sulphate is confined to the blood and that the blood volume is 35% of the body weight (Webb, 1940), then the influx is $0{\cdot}35WM/T$ which in a steady state must equal the outflux. The outflux must be partitioned between diffusion and loss through the urine in the proportion of $B_p/100-B_p$.

Thus sulphate outflux through the excretory organ is

$$\frac{100-B_p}{100}\frac{0{\cdot}35WM}{T},$$

but the outflux must also be $V_u.U_p(M/100)$.

Hence

$$V_u = \frac{100-B_p}{U_p}\frac{0{\cdot}35W}{T},$$

and hence the urine flow as a percentage of the body weight

$$= \frac{100-B_p}{U_p}\frac{35}{T}.$$

B_p and U_p were measured for each animal and the mean value of 5 days for T was used in all cases.

Calculations of the rate of urine production for nine animals from normal sea water are shown in Table 3. The mean value was 3·6% body weight per day. The rate estimated in this way is clearly much lower than that found by Nagel (1934) and slightly lower than found by Webb (1940). It may be noted also that the blood sulphate concentration is well below that found by Webb, although it is similar to the mean value found by Gilbert (1959) in animals collected from the same locality as the present ones.

Table 3. *The rate of urine production in animals from normal sea water*

Crab no.	Blood sulphate concn. (% concn. in medium) (B_p)	Urine sulphate concn. (% concn. in medium) (U_p)	U_p/B_p	Urine production rate (% body weight/day)
21	39·4	131	3·3	3·1
22	40·8	92	2·3	4·3
23	34·0	196	5·8	2·2
24	32·5	187	5·8	2·4
25	34·0	104	3·1	4·2
26	40·9	135	3·3	2·9
27	33·5	143	4·3	3·1
28	25·4	167	6·6	3·0
29	35·0	105·5	3·0	4·1
Means	35·0	140	4·1	3·6
Webb (1940)	57·2	128	2·2	4·7 (2·4)*
Webb (1940) (sea water with increased sulphate)	29·0	139	4·6	(3·6)*

* The figures in parentheses are rates of urine flow calculated from the sulphate concentrations and assuming a time constant for sulphate penetration of 5 days.

Using Webb's values for the sulphate concentration of blood and urine, and the value of the time constant for sulphate penetration as here determined (5 days), the rate of urine production calculated by this method comes out at 2·4% body weight/day for his animals. Thus they must either have had a lower permeability to sulphate than the present ones or Webb overestimated the rate of urine production. He also measured the sulphate concentration in blood and urine of animals kept in sea water with increased sulphate content. From his figures it is clear that the rate of urine production must have been greater than in normal crabs and one may conclude that in crabs from normal sea water the rate of urine production is closely geared to the regulation of the blood sulphate concentration. If this is so then the validity of the weighing method for measuring urine flow is open to question. It seems quite clear that the water required for urine production in *Carcinus* in full-strength sea water is not taken up osmotically since often no osmotic gradient exists and one must suppose, therefore, that the water is absorbed by some active process to an extent which is determined by the blood sulphate level. Now the prevention of sulphate excretion by blocking the excretory pores must lead to an increase in the blood sulphate concentration and, hence, the rate of water absorption would be increased. Thus the weighing method, which measures the rate of water absorption, is likely to give a value for urine flow in excess of normal.

Table 4. *The rate of urine production in animals from dilute sea water*

Crab no.	External medium (% normal sea water)	B_p	U_p	U_p/B_p	Urine production rate (% body weight per day)
30	90	38	69	1·8	6·0
31	80	25	46·3	1·9	10·8
32	75	23·1	46·1	2·0	11·1
33	65	15	16·7	1·1	33·9
34	65	18·1	41·7	2·3	13·1
35	60	20·5	26·5	1·3	19·9
36	50	14·1	35	2·5	16·4
37	50	20·9	33·6	1·6	15·7
38	50	23·1	30	1·3	17·9
39	40	12	17	1·4	34·5
40	40	10·3	17	1·6	35·2
41	40	11·4	17·5	1·5	33·7
42	40	16·6	27·1	1·6	20·5

We can now turn our attention to the rate of urine production in animals adapted to dilute sea water. This was also measured by the sulphate method and the results are shown in Table 4. The rate of urine flow is clearly increased in dilute sea water and reaches a value equivalent to about a third of the body weight per day in animals from 40% sea water. There seems little doubt that this increased rate of flow is due to the large osmotic gradient developed in these animals and it may be noted that here blood sulphate regulation breaks down. The sulphate concentration may be reduced to about one-eighth of that in the blood of normal animals despite the fact

that *U/B* ratio is decreased, in some cases almost to one. The relation between the external concentration and the urine production rate is shown in Fig. 6.

With respect to the maintenance of sodium balance these results show that regulation in dilute sea water is not assisted by urine production; in fact, the reverse is the case. The loss of sodium through the urine is increased in dilute sea water.

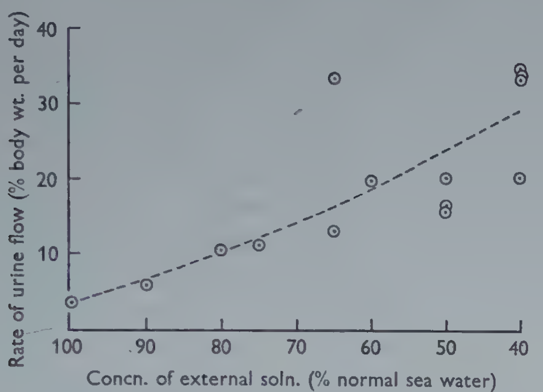

Fig. 6. The relation between the rate of urine production and the concentration of the external solution.

(*d*) *The partition of sodium loss*

Since sodium is lost through both the body surface and the urine, it is necessary to establish the relative importance of the two pathways. This can be readily estimated from the knowledge of the total sodium outflux, the blood sodium concentration and the rate of urine production at a known external concentration. Calculation of the sodium loss in the urine as a percentage of the total sodium loss is shown in Table 5 for animals adapted to normal, 50 and 40% sea water. The mean urine production rate was taken from Fig. 6.

In animals from normal sea water the loss through the urine is negligible compared with the total loss rate. In dilute sea water it is more important but the body surface is still the major route for sodium loss. This is confirmed by the previous measurements of tracer outflux (Table 2). The small decrease in the time constant for sodium loss in dilute sea water can be explained by the additional loss through the excretory organ.

Table 5. *The partition of sodium loss*

External medium (% normal sea water)	Mean blood sodium concn. (mM./l.)	Total outflux for 50 g. animal (μM./hr.)	Mean urine production rate (% body wt./day)	Sodium loss through urine for 50 g. animal (μM./hr.)	Loss through urine as % total outflux
100	460	1267	3·6	34·5	2·7
50	340	1009	23	163	16·1
40	300	891	30	188	21·1

(e) *Sodium influx*

The fact that the permeability of the body surface to sodium is not significantly decreased during adaptation to dilute sea water and that urine production does not assist in the regulatory process means that regulation must be achieved by variations in the rate of active sodium uptake. Before this can be expressed in quantitative terms, however, it is necessary to know more about the properties of the active uptake mechanism and particularly the relation between the rate of uptake and the external sodium concentration.

For the influx measurements the animals were first adapted to 30% sea water so that the uptake mechanism was fully activated. For low external sodium concentration the influx was measured with ^{22}Na. Each crab was placed in the animal chamber of the influx apparatus (Shaw, 1960*a*), together with 100 ml. of water which was circulated through the flow counter. At the start of each experiment the water contained about 5 mM./l. NaCl with added tracer and the decrease of radioactivity of the external solution was recorded over a period of about 2 hr. During this time the animal lost sodium and the external concentration had increased to about 15 mM./l. by the end of the experiment. The net loss rate of sodium during the experiment was calculated from the rate of increase of the external concentration and this value, together with the measured influx, allowed the total loss rate to be calculated. The influx was determined from the rate of decrease of radioactivity in the external solution for the relation: $\ln(y/y_0) = -t/T$, where T was the time constant, and hence the influx $M_{in} = A/T$, where A was the total external sodium content (Shaw, 1959). The results are shown in Table 6. At the range of external

Table 6. *The sodium influx and net loss rate in animals adapted to 30% sea water*

Crab no.	Weight (g.)	Influx time constant (hr.)	External concn. range (mM./l.)	Experimental period (hr.)	Net loss rate (μM./hr.)	Influx range (μM./hr.)	Total loss rate (μM./hr.)
43	31·6	21	4·8–10·5	2·5	228	22·4–50	266
43	31·6	21	11·3–13·6	1·25	184	53·8–64·8	244
44	30	15	10·0–16·0	2·3	174	66·6–166·6	291
45	51·7	9·7	5·4–13·8	1·6	525	55·7–142·3	624
46	73	9·0	5·0–15·7	1·6	654	55·5–174·4	769

concentrations used the influx was small compared with the total sodium loss rate and the animals were not in sodium balance. For reasons explained above (p. 137) it was not possible to extend the influx measurements to higher external concentrations by this method so the influx was calculated indirectly. The same animals were each transferred to small volumes (100 ml.) of sea water at various dilutions below 30% and the net rate of sodium loss was measured over periods of 4–6 hr., from the increase in the external concentration. Since the total loss rate had been determined in the previous experiments it was possible to calculate the influx at the higher external concentrations from the difference between the total loss rate and

the net loss rate. In this way the relation between the sodium influx and the external sodium concentration was determined.

The results for three different animals are shown in Fig. 7. It can be seen that the influx is not proportional to the external concentration and the rate of increase of influx declines as the external concentration is increased.

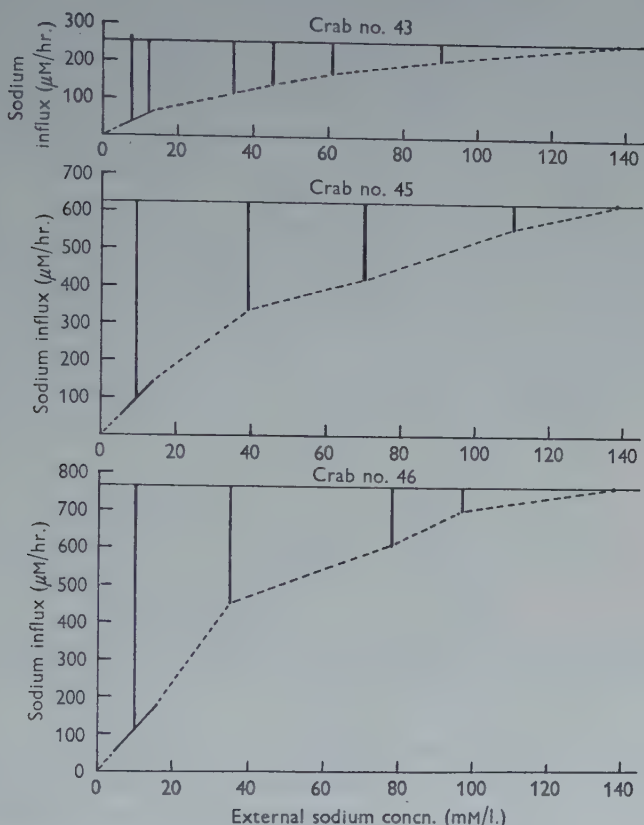

Fig. 7. The relation between the sodium influx and the external sodium concentration in three animals. The dotted line represents the calculated influx. The influx at low external concentrations was measured directly with ^{22}Na. The vertical lines represent the net sodium loss rate.

Now the influx is made up of two components: one due to passive diffusion through the body surface and the other due to the active uptake mechanism. It is important to decide the contribution of each to the total influx. Now at the balance concentration the ratio of the passive component to the total influx must be equal to the ratio of the external sodium concentration to the blood sodium concentration, if we ignore the relatively small loss through the urine. Since these animals were in balance in 30% sea water this ratio is approximately equal to 0·5 (see Fig. 2). Now the passive component of the influx must be proportional to the external concentration so that it is possible to subtract the passive influx from the measured total

influx at all external concentrations and, hence, to derive the relation between the active component of the influx and the external sodium concentration.

This has been done in the case of crab no. 43 using the total influx curve from Fig. 7 and the results are shown in Fig. 8. The upper curve is that of the total influx and the lower that of the passive component of the influx. The vertical distance between the curves represents the active uptake component. It can be seen that the active influx increases rapidly at low external concentrations and then levels off reaching a maximum value at an external concentration of about 70 mM./l. The same result is obtained if the same procedure is adopted for the other animals shown in Fig. 7.

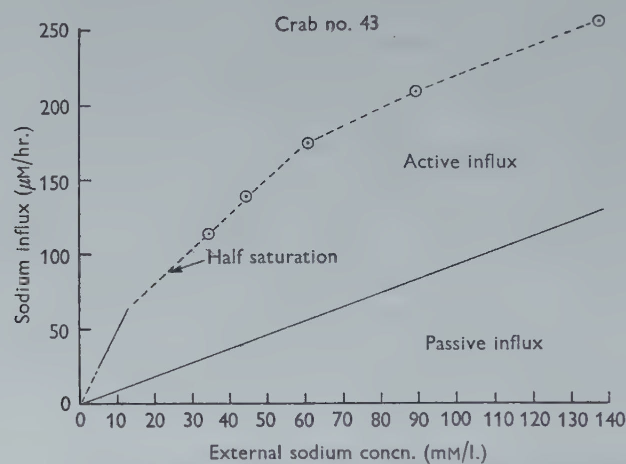

Fig. 8. The relation between the sodium influx and the external sodium concentration in crab no. 43. The influx is divided into active and passive components.

The conclusion is therefore reached that one of the characteristic features of the active uptake mechanism is that it is fully saturated at an external concentration of about 70 mM./l. Now it may be argued that this conclusion is reached by calculations which are admittedly indirect, although no assumptions of doubtful validity have been made. However, the conclusion is fortified by the fact that direct measurements of the active influx in a number of other crustaceans have already led to the same result. Thus the saturable nature of the active uptake mechanisms has been demonstrated in *Astacus pallipes* (Shaw, 1959), *Gammarus pulex* and *G. duebeni* (Shaw & Sutcliffe, 1960) and in *Eriocheir sinensis* (Shaw, 1960*b*). The special feature of the mechanism in *Carcinus* is the relatively high external concentration required for saturation. The external concentration for half-saturation (K_m) is about 20 mM./l. compared with concentrations of between 0·15 and 1·5 mM./l. for the other Crustacea.

From the point of view of sodium balance in *Carcinus* the important feature is that the uptake mechanism is fully saturated at all external concentrations in which the animals can survive, so that in considering the role of the active uptake mechanism

in regulating the blood sodium concentration it is not necessary to consider further the direct effect of the external sodium concentration on the rate of sodium uptake.

(*f*) *The regulatory nature of the active uptake mechanism*

The arguments presented in the previous sections show that the regulation of the blood sodium concentration in *Carcinus* is brought about largely by variations in the rate of active uptake of sodium. Since the uptake mechanism is saturated at all physiological external concentrations then it follows that these variations must result from changes in the maximum rate of transport. This must be the result of different states of activation of the uptake mechanism. The relation between the rate of active uptake and the blood sodium concentration can now be put in quantitative terms.

Referring back to Fig. 2 it is now clear that at any external concentration within the physiological range the rate of active uptake must be proportional to the difference between the concentrations of sodium in the blood and in the medium. This may be expressed as a function of the blood sodium concentration. This has been done in Fig. 9, where the difference between the mean blood sodium concentration and that of the external solution is related to the blood sodium level.

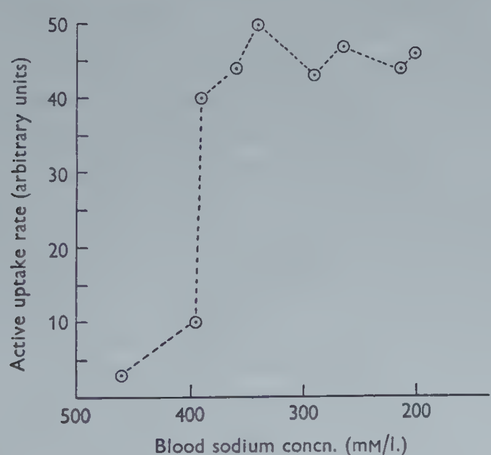

Fig. 9. The relation between the rate of active uptake of sodium and the blood sodium concentration.

The nature of the sodium regulatory mechanism now becomes apparent. Active sodium uptake accounts for the small difference between blood and external sodium concentrations in normal animals as deduced by Webb (1940). The onset of regulation commences with a fall in blood concentration leading to an activation of the uptake mechanism. This is relatively slight until the blood sodium falls to about 400 mM./l. when a small decrease (10–20 mM./l.) below this levels result in the full activation of the uptake mechanism. The rate of transport is increased by a

factor of about fifteen. A further decrease in blood concentration cannot increase the rate of transport beyond this and, hence, from there on the blood concentration must fall in proportion to the external concentration.

DISCUSSION

The results presented in this paper show that the regulation of the blood sodium concentration in *Carcinus* is brought about largely by variations in the rate of active uptake of sodium. No evidence was found of control over the permeability of the body surface and, although the increased rate of urine production in dilute sea water tends to impede regulation, the amount of sodium lost through the urine was never more than about 20% of that lost through the body surface. The properties of the regulating system may therefore be defined by the behaviour of the active uptake mechanism. Two features are particularly worthy of note. The first is that the active uptake rate is a function of the level of sodium in the blood. The precise nature of this relationship determines, in its turn, the level at which the blood sodium is set. Thus a fall in blood sodium concentration of 60 mM./l. from its normal value only induces a small increase in the active uptake rate, but a further small decrease below this fully activates the uptake mechanism. It thus follows that the regulatory mechanism is not so much concerned with the maintenance of the blood sodium concentration at its normal level as with the prevention of a fall in concentration below a certain limit. In terms of the function of the regulatory system as a whole this is of some importance. If a small drop in blood concentration below the normal level led immediately to a full activation of the uptake mechanism then the mechanism would have to be continually turned on and off. Because of the inevitable 'inertia' of the system as a whole, this would lead to the type of instability experienced in a simple on/off thermoregulator when the regulated temperature is only slightly above that of the outside. This kind of response is prevented in *Carcinus* by the fact that the uptake mechanism is only fully operative when the blood concentration is considerably greater than that of the external solution.

The other feature of the uptake mechanism of importance to the regulatory system as a whole is the fact that when fully activated it is limited to a maximum rate of transport. This means that precise regulation of the blood concentration can only be maintained over a relatively small range of external concentrations. When the external concentration falls below a certain value the uptake rate is insufficient to maintain the blood concentration and, thereafter, the blood concentration must fall in proportion to the decrease in external concentration, eventually reaching the lethal level.

Clearly then, for a given surface permeability, the extent to which *Carcinus* can survive in dilute sea water depends on the maximum rate attained by the uptake mechanism when this is fully activated.

It is interesting to compare the sodium regulating system in *Carcinus* with that previously described in *Astacus pallipes* (Shaw, 1959). The two systems have much in common, although they operate at widely different external concentrations. The most important features common to the two are: (*a*) that regulation is effected mainly

by changes in the active uptake rate, and (*b*) that at a critical blood concentration a small decrease leads to a great increase in the uptake rate. The main difference between the two systems lies in the fact that whereas in *Carcinus* the active uptake mechanism is always fully saturated under normal physiological conditions, this is not the case in *Astacus*, although saturation is reached at a much lower external concentration. The significance of this difference seems clear. In *Carcinus* balance is maintained at a high external concentration and, hence, diffusion is a major component of the influx. Thus in animals adapted to dilute sea water an increase in the external concentration leads to an increase in the influx due to the diffusion component and in spite of the fact that the uptake mechanism is saturated. Hence the normal blood concentration is soon restored. On the other hand, in *Astacus* balance is maintained at very low external concentrations and hence diffusion is only a very small component of the total influx. An increase in external concentration within the normal range of concentrations in its environment gives rise to only a very small increase in influx due to this cause. It follows, therefore, that if the uptake mechanism was also saturated at these concentrations then the influx would be largely independent of the external concentration. In fact, at very low external concentrations, the balance point is on the steep part of the influx curve (about one-fifth of the saturation value) so that a small increase in external concentration leads to a substantial increase in the influx and, hence, a rapid restoration of the normal blood concentration.

These considerations emphasize again that in *Astacus*, as well as in *Carcinus*, the regulation of blood sodium, brought about by the activation of the uptake mechanism, is not concerned with the maintenance of the normal blood sodium level as such, but is a protective device which prevents the blood concentration falling below a certain limiting value, which is itself below the normal blood concentration. The mechanism in both cases is adapted to restoring the normal blood concentration under favourable conditions, viz. when the external concentration rises again.

SUMMARY

1. The mechanism of sodium balance in *Carcinus maenas* has been investigated.

2. Measurements of sodium outflux showed no evidence of a decrease in surface permeability to sodium in dilute sea water.

3. The rate of urine production in normal sea water was 3·6% body weight per day and the sodium loss through the urine was insignificant compared with the total sodium loss. In 40% sea water the urine rate was increased to 30% body weight per day and the loss in the urine accounted for 20% of the total loss.

4. Measurements of sodium influx and calculation of the active component showed that the active uptake mechanism was fully saturated at all external concentrations in which the animals could survive.

5. Regulation of the blood sodium concentration is effected largely by the activation of the sodium uptake mechanism. This prevents the blood concentration falling below a critical level as long as the external concentration itself is not too low.

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SODIUM BALANCE IN *ERIOCHEIR SINENSIS* (M. EDW.). THE ADAPTATION OF THE CRUSTACEA TO FRESH WATER

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INTRODUCTION

The main features of the mechanism by which sodium balance is maintained in *Eriocheir sinensis* has already been described by several authors (Krogh, 1938, 1939; Koch, Evans & Schicks, 1954; Koch & Evans, 1956*a*, *b*). Krogh (1938) demonstrated that the animals were freely permeable to salts and that the internal salt content could be rapidly reduced by washing in running distilled water. Further he showed that the inevitable salt loss in fresh water was made good by the active absorption of sodium and chloride ions against a large concentration gradient. Koch, Evans & Schicks (1954) produced evidence that the active process was localized in the branchial epithelium from a study of sodium uptake by the isolated gills. Tracer studies on whole animals (Koch & Evans, 1956*a*, *b*) showed that active uptake of sodium occurred from 1 mM./l. NaCl solutions and a balance between the rate of sodium uptake and loss was found when the external concentration was below 0·5 mM./l.; for some animals being as low as 0·1 mM./l.

The object of the work described in this paper was to obtain a quantitative measure of the uptake and loss rates for animals adapted to external solutions of known concentration. In a single experiment Koch & Evans (1956*a*) reported that an increase in the external concentration from 1 to 2 mM./l. Na led to an increase in the rate of sodium uptake beyond that expected from a linear relation between the two. This was contrary to the results obtained with other freshwater and brackish water Crustacea (Shaw, 1959, 1960 for *Astacus*; Shaw & Sutcliffe, 1961, for *Gammarus duebeni* & *G. pulex*; Shaw, 1961 for *Carcinus*), where the rate of increase of active sodium uptake declined as the external concentration was increased and a saturation level was eventually reached. The relationship between the sodium uptake rate and the external sodium concentration in *Eriocheir* has therefore been examined in more detail.

In the light of the results obtained with *Eriocheir* and those with the other Crustacea mentioned above a hypothesis is advanced to explain the way in which some members of the Crustacea have become adapted to life in fresh water.

MATERIALS AND METHODS

The animals were received by air from Amsterdam and kept in 10% sea water in a laboratory aquarium. For some experiments the animals were previously adapted to 2% sea water, and in certain cases, to lower concentrations.

Sodium influx was measured by means of the sodium isotope, ^{22}Na, in an apparatus similar to that previously described (Shaw, 1960). The animal chamber usually contained 400 ml. of water which was continually circulated through the flow counter. Sodium chloride was added to the water to give the desired sodium concentration and sufficient of the tracer, in the form of NaCl, to give a high initial counting rate. The decrease in radioactivity of the external solution was measured by a recording rate-meter and the influx calculated as before (Shaw, 1959*a*).

The sodium loss rate was determined by placing each animal in 1 l. of de-ionized water and measuring the sodium concentration of the water at intervals by means of an EEL flame photometer. The initial rate of increase in concentration of the water was used to calculate the rate of sodium loss from the animal.

RESULTS

(*a*) *Sodium loss rate*

The rate of loss of sodium into de-ionized water for five different individuals is shown in Table 1. The mean rate of loss was 323 μM./hr./153 g. body weight. This agrees with the single determination of loss rate made by Krogh (1938). He found that an animal weighing 144 g. lost Cl (and Na) at a rate of 200–400 μM./hr. These measurements also support Krogh's contention that the bulk of the salt loss occurs through the body surface rather than through the excretory organ. In an experiment where the openings of the antennary gland were alternately open and closed, he found that the rate of chloride loss showed regular alternations between 108 and 82 μM./hr. showing that only about 24% of the Cl loss took place through the excretory organ. Scholles (1933) found that the rate of urine production in *Eriocheir* was about 4% of the body weight per day. Thus, assuming a blood sodium concentration of 280 mM./l., a 153 g. animal would lose 1714 μM./Na/day (71 μM./hr.) by this means. Since the total loss rate is 323 μM./hr. (Table 1) the loss through the urine accounts for 22% of the total loss, a figure very similar to that found by Krogh.

Table 1. *The sodium loss rate in* Eriocheir sinensis

Crab no.	Body weight (g.)	Sodium loss rate (μM./hr.)	Specific loss rate (μM./hr./153 g. body wt.)
1	124	176	273
2	140	370	404
3	171	346	310
4	161	360	342
6	167	314	288
Mean	153	—	323

(*b*) *Sodium influx*

The relation between the sodium influx and the external sodium concentration is shown in Fig. 1. The relationship is similar to that found in *Astacus pallipes* (Shaw, 1959*a*, 1960), *Gammarus pulex*, *G. duebeni* (Shaw & Sutcliffe, 1961) and *Carcinus maenas* (Shaw, 1961). It is non-linear and the influx tends towards a maximum rate indicating saturation of the transport system at the higher external concentration. Saturation is reached at an external concentration of about 4 mM./l. Na. In detail the relation differs from that of the truly freshwater species, like *Astacus* and *Gammarus pulex* in which saturation is reached at much lower concentrations. It is very similar to that of *G. duebeni* and, in fact, from the physiological point of view the two species have much in common. Thus both are brackish water animals which maintain a high blood concentration and which can just survive in external concentrations down to about 0·2 mM./l.

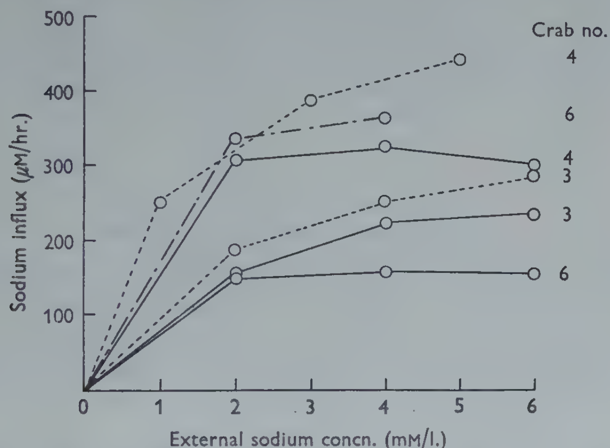

Fig. 1. The relation between the sodium influx and the external sodium concentration in *Eriocheir sinensis*. O—O represent measurements on animals adapted to 10% sea water; O----O adapted to 2% sea water; and O -—-O adapted to 2 mM./l. NaCl.

A comparison between the loss rate and the influx measured at an external concentration at or near the concentration at which the animals were in balance is shown in Table 2. In all cases there was a reasonable agreement between the two values and, thus, there is no reason to doubt that the measurement of the sodium influx by the tracer method gives a close approximation to the true sodium uptake rate.

Measurements of the sodium influx from a 4 mM./l. NaCl solution for animals first adapted to 10% sea water and then to 2% sea water are shown in Table 3. Adaptation to the lower concentration was associated with a higher influx which was especially marked in one case. It is probable, therefore, that the mechanism of adaptation to lower concentration involves, in part at least, the activation of the sodium uptake mechanism. Activation of the uptake mechanisms has been shown previously to occur in *Astacus*, *Gammarus pulex* and *G. duebeni* and *Carcinus*.

Table 2. *A comparison of the sodium influx and loss rate at concentrations at or near the balance point*

Crab. no.	Adapted to	Concn. at which sodium influx was measured (mM./l.)	Sodium influx (μM./hr.)	Sodium loss rate (μM./hr.)
1	1 mM./l. NaCl	1·0	222	176
3	2% sea water	6·0	285	346
4	2% sea water	6·0	444	360
6	2 mM./l. NaCl	2·0	333	314

Table 3. *The effect of adaptation from 10% to 2% sea water on the sodium influx*

Crab no.	Sodium influx from 4 mM./l. NaCl (μM./hr.): Animals adapted to 10% sea water	Sodium influx from 4 mM./l. NaCl (μM./hr.): Animals adapted to 2% sea water	Influx ratio
3	222	250	1·1
4	326	410	1·2
6	158	363	2·3

(*c*) *Summary of balance conditions*

The results presented in the previous sections show that in external concentrations down to about 2% sea water the active uptake mechanism is fully saturated and the rate of uptake just balances the rate of loss which occurs primarily through the body surface. If the external concentration falls below that required for saturation, then balance may be achieved at least in part by the activation of the uptake mechanism.

THE ADAPTATION OF THE CRUSTACEA TO FRESH WATER

The mechanism of sodium balance has now been described for *Carcinus maenas* from brackish water, for *Eriocheir sinensis* and *Gammarus duebeni* from brackish to fresh water and for *Potamon niloticus*, *Astacus pallipes* and *Gammarus pulex* from fresh water. It is now proposed to attempt to draw some general conclusions on the basis of these studies which point to the factors involved in the adaptation of the Crustacea to fresh water.

In a previous paper (Shaw, 1959*b*) one of these factors, viz. the progressive reduction in the permeability of the body surface, has been discussed. The importance of this was previously stressed by Nagel (1934) as a result of studies on the rate of iodide penetration into a number of decapod Crustacea from different types of environment. Although iodide penetration can only be used in a rough qualitative manner as a measure of salt permeability there is no doubt that Nagel was right in his general conclusion and this has now been confirmed by direct measurements

of the rate of sodium loss. Measurements of the sodium loss rate in four species of decapod Crustacea are shown in Table 4. A direct comparison of the values is complicated by the difference in the mean weight between the species. To facilitate comparison the specific loss rate has been calculated in two ways. The first was based on the assumption that the loss rate is proportional to the body weight. This assumption may not be justified. Since the loss rate is controlled by the surface permeability it is possible that it is more closely related to the surface area than to the body weight and, hence, the 'two-thirds' rule might be more applicable. Specific loss rates calculated from this relation are also shown in Table 4. Whichever method of calculation is used, a comparison of the loss rates leads to the same general conclusion. Compared with *Carcinus*, *Eriocheir* shows a considerable reduction in the loss rate and this is still further reduced in the freshwater species. The reduction can only be accounted for to a small extent by the reduction in the blood sodium concentration, hence the main factor must be a decrease in the permeability of the body surface to sodium. The work of Nagel (1934) and Margaria (1931) showed that the marine decapod Crustacea are more permeable than *Carcinus*, hence in passing from a marine to a freshwater environment these animals clearly display a progressive reduction in the permeability of the body surface to salts.

Table 4. *Sodium loss rate in several decapod Crustacea from different environments*

Species	Habitat	Mean body wt. (g.)	Mean blood Na concn. (mM./l.)	Sodium loss rate (µM./hr.)	Specific sodium loss rate (proportional) (µM./hr./50 g.)	Specific sodium loss rate ('two-thirds' rule) (µM./hr./50 g.)	Reference
s maenas (40% sea water)	Brackish water	50	300	891	891	891	Shaw (1961)
ir sinensis (from sea water)	Brackish to fresh water	153	280 (estimated)	323	102	153	This paper
n niloticus	Fresh water	15	259	12	40	27	Shaw (1959*b*)
pallipes	Fresh water	12	186	1·8	7·5	4·7	Shaw (1959*a*)

The other factor of importance which has emerged from the studies on sodium balance is concerned with the characteristic properties of the sodium uptake mechanisms displayed by animals from different environments. In all the animals the sodium uptake mechanism was found to be saturated at high external concentrations but the value of the external concentration at which saturation occurred varied in the different species. For each species the uptake mechanism can be characterized by a particular external concentration at which the mechanism is half-saturated and this is shown in Table 5. A comparison of the half-saturation values shows that a brackish water species, such as *Carcinus*, is characterized by the possession of an uptake mechanism with a relatively low affinity for sodium whereas the mechanisms of the freshwater species have a very high affinity for the ion.

Comparing *Carcinus* with *Eriocheir* and *Gammarus duebeni* it can be seen that the half-saturation concentration is reduced by about ten times and this is reduced about ten times again in the truly freshwater species. Thus the second factor involves a reduction in the external concentration at which saturation of the active uptake mechanism occurs.

Table 5. *The values of the external concentration for half-saturation of the sodium uptake mechanism in Crustacea from different environments*

Species	Habitat	Ext. concn. for half-saturation of the uptake mechanism (mM./l. Na)	Reference
Carcinus maenas	Brackish water	*ca.* 20	Shaw (1960*b*)
Eriocheir sinensis	Brackish to fresh water	*ca.* 1·0	This paper
Gammarus duebeni	Brackish to fresh water	1·5	Shaw & Sutcliffe (1960)
Astacus pallipes	Fresh water	0·2–0·3	Shaw (1959, 1960)
Gammarus pulex	Fresh water	0·15	Shaw & Sutcliffe (1960)

The way in which these two factors interact can be seen by considering the effect of a reduction in surface permeability on the external concentration required for the maintenance of sodium balance in two animals which possess a high and low affinity uptake mechanism respectively.

Sodium balance is maintained when the sodium influx just equals the outflux. Now the influx is made up of two components—one due to active uptake and the other due to passive diffusion. Thus balance is maintained when the active uptake rate is equal to the difference between the outflux and the passive influx. Now if we ignore the relatively small losses of sodium through the urine—as has been found in *Eriocheir*, *Carcinus* (Shaw, 1960*b*) and in *Astacus* (Bryan, 1960)—then the difference between the outflux and the passive influx (the net passive loss rate) is proportional to the difference between the sodium concentration of the blood and the external solution.

Hence the net passive loss rate $(L) = K_1(B-C)$, where K_1 is a constant relating to the permeability of the body surface, B is the blood sodium concentration and C, the sodium concentration of the external solution.

Now the active uptake rate (U) is also a function of C. It has been found that the Michaelis equation in the form: $U = K_2[C/(K_m+C)]$, where K_2 is the maximum uptake rate and K_m, the external concentration for half-saturation, gives a reasonable approximation of this relation (Shaw, 1959*a*; Shaw & Sutcliffe, 1960).

Hence balance is maintained when $K_2[C/(K_m+C)] = K_1(B-C)$. Now consider two animals with uptake mechanisms having the same maximum rate of uptake (K_2), but widely different values of K_m (such as is the case in *Carcinus* and *Eriocheir*), and consider the effect of reducing the permeability of the body surface (i.e. reducing K_1). The situation is shown graphically in Fig. 2, where animal 1 has a K_m value of 40 mM./l. and animal 2, a value of 2·5 mM./l. The blood sodium concentration in both is 300 mM./l.

Consider first the case where the surface permeability is relatively high (e.g. $K_1 = 32$, in Fig. 2). In the case of animal 1 balance is maintained at an external concentration of 180 mM./l. In animal 2, despite the low value of K_m the balance concentration is only slightly lower (150 mM./l.). As the permeability (K_1) is reduced the difference becomes much more pronounced. Thus if the permeability is halved (i.e. $K_1 = 16$) then for animal 1 the balance point is at 90 mM./l., whereas for animal 2 it is only 25 mM./l. If the permeability is halved again ($K_1 = 8$) then animal 1 balances at an external concentration of 32·5 mM./l., whereas animal 2 requires only 2·5 mM./l. To achieve balance at this concentration animal 1 would have to reduce its permeability by a further factor of 8. To be in sodium balance at the lowest concentration found for freshwater animals (about 0·05 mM./l., Shaw, 1959*a*, *b*) animal 1 would have to achieve an over-all reduction in permeability of about 1600 times, whereas an animal with a K_m value of 0·2 mM./l. could do this with a permeability reduction of only 8 times.

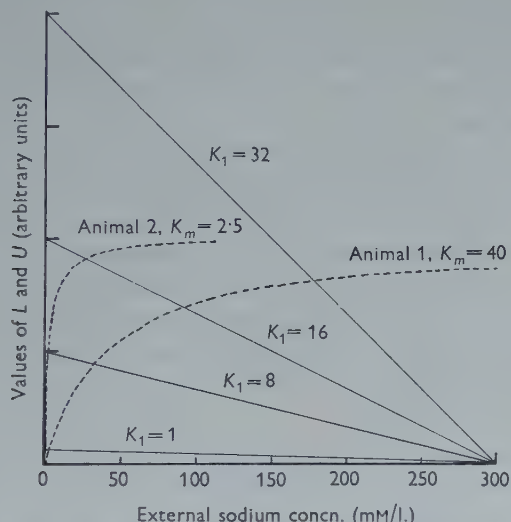

Fig. 2. The balance conditions for two hypothetical animals maintaining the same blood sodium concentration (300 mM./l.). The straight lines represent the net passive loss rate (L) according to the relation: $L = K\,(B-C)$ for different values of K_1. The dotted lines represent the active uptake rate (U) according to the relation: $U = K_2\,(C[(K_m+C)]$ for the two animals. For animal 1, $K_m = 40$ mM./l. and for animal 2, $K_m = 2{\cdot}5$ mM./l. Both animals have the same maximum uptake rate (K_2).

Thus the acquisition of a high-affinity uptake mechanism overcomes the necessity of an excessive reduction in permeability to maintain balance at the low concentrations found in fresh water. In addition, the uptake mechanism is able to work at or near saturation point which is probably the most efficient position.

It is therefore suggested that the adaptation of the Crustacea to fresh water involves two main factors which together allow balance to be maintained at very low concentrations. The first is the gradual reduction in the permeability of the body

surface to salts, and the second, a reduction in the external concentration at which saturation of the active uptake mechanism is reached. The latter is brought about by the fact that the uptake mechanism acquires a higher affinity for the ions which it transports.

This hypothesis may now be considered in relation to other theories on the adaptation of the Crustacea to fresh water. Beadle & Cragg (1940) suggested that adaptation has proceeded by two main stages: (*a*) the maintenance of a high blood concentration in fresh water probably by active ion absorption, and (*b*) the evolution of a renal salt-reabsorption mechanism and a lowering of the blood concentration. It can be seen that the present hypothesis is concerned with the mechanism by which Beadle & Cragg's first stage is achieved. The two views are thus complementary, the only difference being one of emphasis. Thus the acquisition of a low surface permeability and a high-affinity uptake mechanism is regarded here not as a stage in the adaptation but as the primary process by which the invasion of fresh water is accomplished. Many animals may stop here—an example is *Potamon niloticus*. The acquisition of a low blood concentration and the production of a dilute urine are not obligatory but may be regarded as additional refinements which may or may not be present. Even if they are not present the animals may nevertheless be well adapted to freshwater conditions.

Potts (1954) considered the problem from the point of view of the energy requirements for osmoregulation and concluded that the most important factors involved were a reduction in blood concentration and the possession of a renal salt-absorbing mechanism (i.e. those factors regarded as refinements in the present view). However, his conclusions were based on the assumption that the animals were semi-permeable even in the brackish water phase. The validity of this assumption was questioned previously (Shaw, 1959*b*) and has now been shown to be incorrect in the case of *Carcinus* (Shaw, 1961), *Eriocheir* and *Potamon niloticus* (Shaw, 1959*b*).

However the fact remains that some of the freshwater Crustacea do produce a dilute urine and show a marked reduction in blood concentration and the significance of this must be considered.

It was pointed out previously (Shaw, 1959*b*) that these factors are of especial importance in reducing the osmotic work done in osmoregulation, as Potts suggested, in the special case where the animal had already achieved a differential reduction in permeability to salts but not to water and, hence, become essentially semi-permeable. It now seems probable that this condition will prove the exception rather than the rule amongst the freshwater Crustacea.

The development of the ability to produce a dilute urine may have occurred in some animals for other reasons. Thus one feature of many freshwater animals is the fact that they can only withstand a small increase in blood concentration. If the animals can produce a dilute urine and can concentrate it in response to a rise in blood concentration then this provides them with an additional mechanism for the control of the blood concentration. Such a mechanism might be of considerable functional importance.

On the question of the reduction in blood concentration, Beadle & Cragg stressed the importance of this factor in reducing the blood/tissue Cl gradient to levels more easily maintained. This may well be the case, although in *Carcinus maenas*, when the blood/muscle fibre Cl gradient was examined under conditions of reduced blood concentration it was found that the normal gradient was maintained (Shaw, 1955). It seems more likely that the main value of a reduction in blood concentration lies simply in the fact that the concentration of ions in the blood determines their rate of movement. Thus a reduction in the NaCl concentration of the blood reduces the rate of salt loss from the animal as a whole (and, hence, acts together with a reduction of surface permeability in producing conditions favourable to the maintenance of salt balance in low external concentrations) and also reduces the rate of entry of NaCl into the tissue cells. Since the cellular ionic balance is maintained by the active extrusion of ions at a rate which must balance their rate of entry, clearly a reduction in the latter decreases the osmotic work which must be done at the cell surfaces.

SUMMARY

1. In *Eriocheir sinensis* active uptake of sodium plays a vital role in the maintenance of sodium balance. At external concentrations down to about 6 mM./l. the active uptake mechanism is fully saturated and the uptake rate just balances the rate of loss, which occurs primarily through the body surface. At lower external concentrations balance may be achieved, at least in part, by the activation of the uptake mechanism.

2. A hypothesis is put forward to account for the mechanism of adaptation of the Crustacea to fresh water. Two main factors are involved: (*a*) a progressive reduction in the permeability of the body surface to salts and, (*b*) the acquisition of an active uptake mechanism with a high affinity for the ions which it transports.

3. This hypothesis is discussed in relation to previous theories on the adaptation of the Crustacea to fresh water.

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FUEL UTILIZATION AND DURATION OF TETHERED FLIGHT IN *APHIS FABAE* SCOP.

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(*Received* 12 *August* 1960)

INTRODUCTION

The objects of this work were to determine the nature and amounts of fuel used during flight in *Aphis fabae* Scop., their histological distribution and their relation to the duration of tethered flight. The aphids were flown suspended in an air-stream of a speed equivalent to their own flight speed. How the metabolic rates of aphids flying in this manner compare with those during free flight is not known, but the quantitative results are comparable with those obtained for tethered flight of some other insect species (see Wigglesworth, 1949; Weis-Fogh, 1952; Hocking, 1953).

MATERIAL

Alate alienicolae and migrantes from laboratory culture or natural infestations were used.

Aphids in the laboratory were bred at 19 ± 5° C. on broad beans (*Vicia faba* L., var. Claudia Aquadulce). For experiments, alienicolae from healthy colonies of the same clone were transferred to new plants $\frac{1}{4}$–$\frac{1}{2}$ hr. after the final ecdysis.

Aphids from natural infestations were from different plants; migrantes from spindle (*Euonymus europaeus* L.), alienicolae from curled dock (*Rumex crispus* L.), broad bean (*Vicia faba* L., Claudia Aquadulce) and winter bean (*Vicia faba* L., Garton's S.Q.). Parts of these plants with 4th-instar alate nymphs were cut off and stood in water in the laboratory; as ecdysis occurred the adults were transferred to small bean plants.

All aphids were kept in the dark at 20° C. for 24 ± 1 hr.; thus they had been flight mature for about 11 hr. when used in the experiments (Taylor, 1957).

METHODS

General procedure

Of the nine main experiments, nos. 1–5 assessed fat consumption during flight and 6–9 glycogen consumption. Fat and glycogen contents of unflown (control) and flown aphids were estimated chemically. To eliminate variations in fuel content caused by host condition or aphid age, control and flown insects in each experiment were from the same plant and of the same age (± 1 hr.).

Initial fat contents of flown aphids were found by interpolation of graphs relating fat content to live weight in the controls. The differences between these values and

the observed final fat contents represented the amounts used. To assess the amount of glycogen consumed, absolute glycogen contents of flown and unflown insects were compared.

Weighing methods

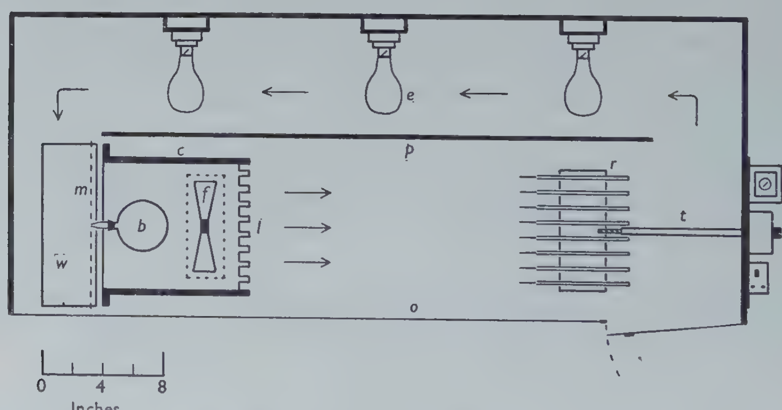
(*a*) *Individual aphids.* Aphids to be flown in Expts. 1–5 were temporarily immobilized by cooling, and the mean of three successive weighings (to 0·01 mg.) taken. Each aphid was then placed in a numbered tube in darkness at 15–17° C. and allowed ½–1 hr. to recover before being tethered for flight. Control aphids were killed with ethyl acetate vapour and immediately weighed and grouped into batches of ascending weight.

(*b*) *Batches of aphids.* Controls in Expts. 6–9 and flown aphids in Expts. 1–9 were weighed in batches immediately after exposure to ethyl acetate vapour.

Flying method

The aphids were tethered for flight by attaching them by the dorsum of the thorax to fine pins with water-colour paint (see Johnson, 1958). All experiments were done in a flight chamber (Text-fig. 1), illuminated from above and heated by nine 60 W. electric lamps. A 6 in. Vent-Axia fan circulated air within the chamber and gave an air current of 1 m.p.h. in the working section. Temperature was controlled at 25–26° C. by a Sunvic thermostat and humidity maintained at 57–82 % R.H. by an evaporator at the fan intake. The pin holders were placed on a three-tiered rack.

Five to twenty aphids were flown simultaneously facing into the air stream; those repeatedly refusing to fly during the first ½ hr. were rejected.



Text-fig. 1. Plan of the flight chamber: *b*, water bottle; *c*, fan casing; *e*, electric lamp; *f*, fan; *l*, louvres; *m*, muslin sheet; *o*, observation panel; *p*, partition; *r*, rack with mounting pins; *t*, thermostat; *w*, water bath.

Analytical methods

(*a*) *Fat determination.* 'Fat', estimated as total ether extractive, includes neutral fats, fatty acids and other ether-soluble material, some of which is valueless as energy reserve.

Batches of aphids were dried at 70° C. to constant weight and then kept at 38° C. for 48 hr. in extraction tubes with 5 ml. petroleum ether (b.p. 40–60° C.) which was

changed approximately every 6 hr. The insects were then dried and re-weighed. The weight of ether-soluble material was thus given by subtraction, not by direct weighing of the extract. Increasing the extraction time to 96 hr. did not increase the amount extracted.

The insects were kept intact by transferring them with a single hair and changing the solvent with a micro-pipette; error from loss of appendage parts was negligible. A minimum of 0·2 mg. fat was aimed at for each determination in unflown insects. Ten aphids were therefore used in each control batch and wherever possible in the flown batches.

(*b*) *Glycogen determination.* Glycogen was estimated by the method of Seifter, Dayton, Novic & Muntwyler (1950) for tissues of low glycogen content, modified as follows: (1) After digestion in 30% KOH the material was filtered through a micro-sintered glass filter (porosity 5–10 μ) and the residue washed in a further quantity of KOH. Filtration was required because of much insoluble material which could not be centrifuged down. (2) The sedimented glycogen was dissolved in 1 ml. distilled water to which 2 ml. of anthrone reagent were added. Thus, a reaction mixture of 3 ml. was tested as against 15 ml. in the original method.

Amounts of chitin similar to that in the insects gave no visible colour when treated and no absorption at 620 mμ, so there was no interference from chitin. As a check on glycogen loss, however, known amounts of glycogen were taken through the same procedure. Three experiments at each of five glycogen levels (0·01–0·05 mg.) gave recoveries of 80·0–94·0%, with a mean of 85·5%. Most of the 14·5% loss probably occurred during filtration and in drainage of the glycogen precipitate; the results of analyses of aphids were corrected for a loss of this magnitude.

The method was sensitive enough (practical range under above conditions 0·002–0·05 mg.) to allow batches of 5 aphids to be used. However, more were usually taken, i.e. 5, 10 or 20 in each control and about 10 in each flown batch.

Histochemical methods

All insects were decapitated before fixation. To demonstrate glycogen, insects were fixed in Carnoy's, Bouin's or Bouin–Doboscq's fluid, embedded in paraffin and celloidin, and sectioned at 8 μ. Sections were stained in Lugol's solution, Best's carmine, or by the periodic acid-Schiff technique (see Carleton & Drury, 1957). For fats, the insects were fixed in Baker's formaldehyde-calcium, embedded in gelatine and sectioned at 15 μ. Sections were stained in Sudan Black B.

Mass of the flight muscles

It is impracticable to dissect out and weigh the flight muscles of *Aphis fabae*, so the following method was adopted using unflown 24 hr. old alatae from culture. Aphids were serially sectioned and the area occupied by muscle and other tissues in each section of the thorax measured; the volume of muscle as a percentage of the total thoracic volume was then computed. The thoraces of other aphids were isolated and weighed. The weight of the muscles was then calculated using the volume percentage as a weight percentage, assuming that the density of muscle is of the same order as that of other thoracic tissues.

The calculated muscle weights are possibly too high, because the muscle density is

less than that of the rest of the thorax, which includes cuticle. The high density of the cuticle, however, will tend to be counterbalanced by the low density of the fat-body cells. The method is probably at least as accurate as that used by Chadwick & Gilmour (1940) to determine flight-muscle weight in *Drosophila*—by subtracting the dry weight of the thoracic skeleton, after treatment with KOH, from the weight of the freshly killed thorax.

RESULTS

Flight performances

Different aphids behaved differently during tethered flight. Some stopped flying for no apparent reason, only to re-start within a few seconds; others stopped occasionally and required re-stimulation; others flew continuously for periods of 5–8 hr. Those aphids that stopped and failed to re-start spontaneously were induced to fly again by stimulating their tarsi, or, by passing them rapidly forward through the air.

Aphids were flown for set times of up to 6 hr. in Expts. 1–9, and to apparent exhaustion in Expts. 10 and 11. In the latter experiments, flight usually became intermittent as the aphids became fatigued, but there was generally no sharp end-point to flight (cf. Johnson, 1955). An aphid was considered exhausted therefore when it ceased to fly, and: (1) it could not be induced to fly again, even after a rest of *c.* 1 min. (39 % of the insects flown in Expts. 10 and 11); or (2) stimulation produced only a mere vibration of the wings (5 %); or (3) after further stimulation, three successive flights each lasted less than 1 min. (56 %).

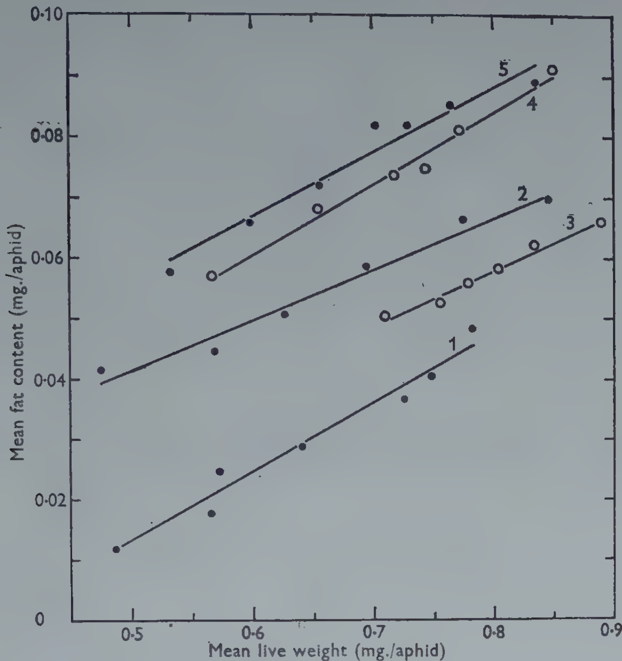
Fuel content before flight

(*a*) *Fat content.* The 24 hr. old unflown aphids reared in the laboratory on broad bean contained least fat (Expt. 1), the mean proportion of fat to live weight being 4·4 % (15·6 % of the dry weight). Aphids from a natural infestation of a field bean (Expt. 5) contained most, with 11·1 % of the live weight (31·4 % of the dry weight). Within each experiment the mean weight of fat per aphid was highly correlated with live weight; separate regressions for the two variables were fitted for each series of controls (Text-fig. 2). On the average, positive differences of 0·1 mg. live weight were associated with positive differences of 0·008–0·012 mg. fat/aphid. The positions of the regression lines demonstrate the different amounts of fat in the insects from the various hosts, differences that probably reflect the nutritional value of the sap imbibed by the aphids during development.

(*b*) *Glycogen content.* The aphids analysed for glycogen were from culture on broad beans (Expts. 6–9). The glycogen constituted between 0·5 and 1·0 % of the live weight of the insects (1·7–3·4 % of the dry weight). Glycogen content and aphid live weight were not significantly correlated ($r = 0{\cdot}066$; $P > 0{\cdot}10$) (Text-fig. 3).

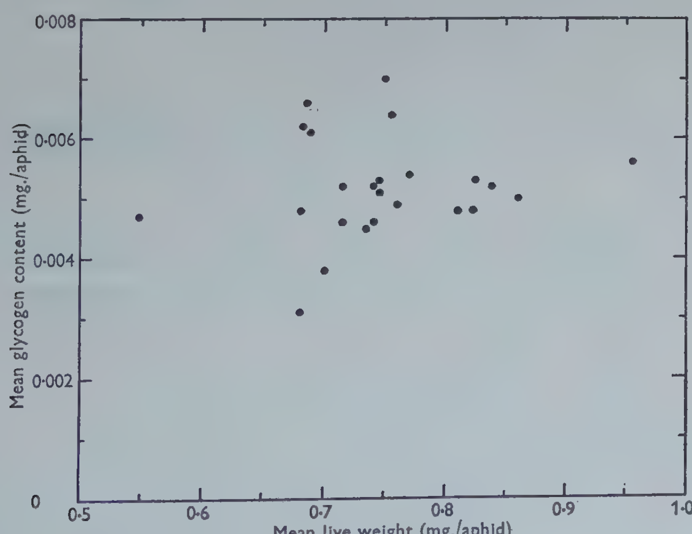
Fuel utilization during flight

(*a*) *Fat utilization.* Table 1 gives the mean fat contents of batches of aphids before and after flight: pre-flight fat contents were estimated by interpolation of the appropriate regression in Text-fig. 2 (mean wt. of fat/aphid); pre- and post-flight live weights are given elsewhere (Table 2, Cockbain, 1961). Differences between initial and final fat represents the amounts used during flight.



Text-fig. 2. Relationship between mean fat content and live weight in unflown 24 hr. old alatae from different host plants:

Expt. no.	Host plant	Location	Corr. coeff. (*r*)	Reg. coeff. (*b*)
1	Broad bean	Culture	+0·968	+0·115
2	Spindle	Field	+0·986	+0·084
3	Dock	Field	+0·989	+0·092
4	Broad bean	Field	+0·985	+0·118
5	Field bean	Field	+0·972	+0·106



Text-fig. 3. Relationship between mean glycogen content and live weight in unflown 24 hr. old alatae.

Table 1. *Fat contents of batches of aphids before and after flight (Expts.* 1–5)

Flight duration (hr.)	Aphid condition	Expt. 1 No. of aphids	Expt. 1 Weight of fat (mg.)	Expt. 2 No. of aphids	Expt. 2 Weight of fat (mg.)	Expt. 3 No. of aphids	Expt. 3 Weight of fat (mg.)	Expt. 4 No. of aphids	Expt. 4 Weight of fat (mg.)	Expt. 5 No. of aphids	Expt. 5 Weight of fat (mg.)
1	Before flight	10	0·24	9	0·50	8	0·50	7	0·54	11	0·8[illegible]
	After flight	10	0·23	9	0·52	8	0·475	7	0·51	11	0·8[illegible]
2	Before flight	8	0·15	8	0·43	9	0·52	8	0·64	8	0·6[illegible]
	After flight	8	0·12	8	0·365	9	0·45	8	0·535	8	0·5[illegible]
4	Before flight	10	0·30	8	0·44	9	0·54	8	0·66	8	0·6[illegible]
	After flight	10	0·17	8	0·345	9	0·38	8	0·505	8	0·4[illegible]
6	Before flight	8	0·30	7	0·47	9	0·53	7	0·57	7	0·5[illegible]
	After flight	8	0·125	7	0·295	9	0·23	7	0·365	7	0·4[illegible]

Table 2. *Analysis of variance of mean decreases in fat content during flights of different duration (Expts.* 1–5)

Flight duration (hr.)	1	2	4	6
Mean decrease (mg. fat/aphid) (±0·00103) ...	0·0010	0·0080	0·0156	0·0260

Source of variance	D.F.	Mean square
Between hosts (expts.)	4	0·0000353**
Between flight durations	3	0·0005738***
Residual	12	0·0000053

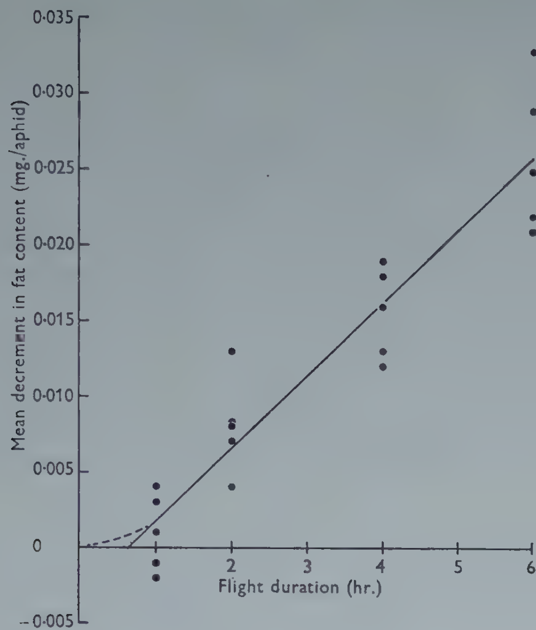
** P = 0·01–0·001. *** P < 0·001.

Decreases in fat, recorded in all but two of the batches, represented 27–58 % of the initial amount in aphids flown for 6 hr. The analysis of variance (Table 2) shows that no significant decrease had occurred after a flight of 1 hr., but the mean decreases after 2, 4 and 6 hr. were all significant. The significant difference between the amounts of fat used in the different experiments, shown by the analysis, was possibly caused by differences in insect weight and 'flight intensity'.

Text-fig. 4 shows mean estimated decrements in fat content during flight. After the first hour, the average rate of fat consumption was 0·005 mg./aphid/hr. The absence of a significant decrease during the first hour, and the fact that the regression line cuts the abscissa before the origin, suggests that a different fuel was used during the early period of flight.

(*b*) *Glycogen utilization.* Text-fig. 5 (graph A) shows the mean glycogen contents of aphids after flights of $\frac{1}{4}$–5 hr. The curve fitted to the mean values shows a high rate of loss during early flight, with a mean decrement of 0·0028 mg./aphid during the first hour and 0·0006 mg. during the second. The decrease during flight was highly significant, a significant decrease being recorded even after $\frac{1}{2}$ hr. (Table 3).

(*c*) *Effect of excretion.* The frequency of excretion of a batch of aphids during flight follows a course similar to the rate of glycogen decrease, i.e. it is highest early in flight. In view of possible interference from gut carbohydrates in the glycogen estimations (anthrone procedure), the possibility of errors from excretion were tested. The number of honeydew droplets excreted during flight and the post-flight glycogen contents of the flown aphids were compared with those of aphids starved (without flight) for equivalent periods at the same temperature (Table 4 and Text-fig. 5,



Text-fig. 4. Decrease in fat content during flight at 25–26° C. $b = +0{\cdot}0048$.

Table 3. *Analysis of mean decreases in glycogen content during flights of different duration* (*Expts.* 6–9)

Flight duration (hr.)	0	¼	½	1	2	3	5
Mean glycogen content (mg./aphid)	0·0052	0·0041	0·0036	0·0024	0·0018	0·0010	0·0009
S.E.	±0·00017	—	—	±0·00041	—	—	—
Mean decrease (mg. glycogen/aphid)*	—	0·0011	0·0016	0·0028	0·0034	0·0042	0·0042
S.E.	—	—	—	±0·00044	—	—	—

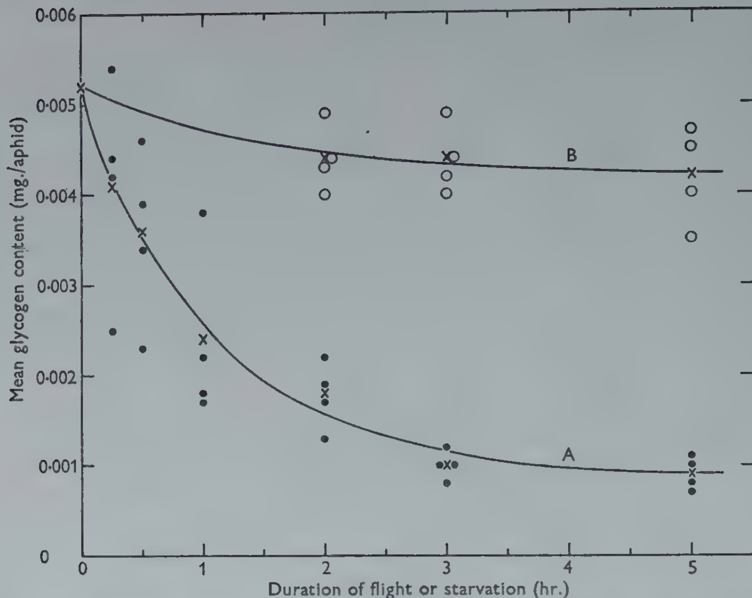
* Decrease significant ($P = 0{\cdot}02$) when greater than 0·0013, i.e. 0·00044 × 3.

graph B). Excretory droplets were collected on pH indicator paper. Droplet sizes were similar in both flight and starvation experiments.

Starved aphids excreted more than flying aphids but their glycogen contents decreased only slightly. The mean decrease during 5 hr. (0·001 mg./aphid) was similar to that which occurred during a ¼ hr. flight and was probably brought about by muscular activity, e.g. walking, during the experiment. Thus the diminution in glycogen during flight represented the amounts utilized.

Rates of metabolism during flight

Approximate metabolic rates were calculated from the mean amounts of fat used in Expts. 1–5 (Text-fig. 4) and glycogen in Expts. 6–9 (Text-fig. 5) using the conversion figures given by Carpenter (1939); the two batches in which there was no decrease in fat have been omitted from the calculations. The rates are given in Table 5 with the calculated contributions of fat and glycogen to the total amount of energy transformed.



Text-fig. 5. Glycogen contents after different durations of (A) flight and (B) starvation at 25–26° C. ×, mean values.

Table 4. *Excretory activity of alatae during flight and starvation at 25–26° C.*

Period of flight or starvation (hr.)	Flight		Starvation	
	No. of aphids	Droplets/aphid	No. of aphids	Droplets/aphid
2	38	0·79	30	1·43
3	36	0·86	30	1·57
5	37	0·97	20	1·75

Table 5. *Rates of metabolism during tethered flight and contributions of fat and glycogen to the total amount of energy transformed*

Flight duration (hr.)	Metabolic rate		Energy derivation	
	Energy expenditure (cal./g./hr.)	Oxygen consumption (ml./g./hr.)	From glycogen (%)	From fat (%)
1	52·2	10·8	30	70
2	65·9	13·8	16	84
4	59·5	12·5	10	90
6	57·7	12·2	7	93

The mean rate was 59 cal./g. live wt./hr. or 12 ml. O_2/g./hr. At this rate, the maximum amount of glycogen in the aphids from culture would maintain flight for 42 min., i.e. 0·001 mg. glycogen if used exclusively would maintain a 6 min. flight; the mean flight duration of *Drosophila melanogaster* on 0·001 mg. glucose is 6·3 min. (Wigglesworth, 1949). Thus over 8 times the maximum amount of glycogen so far recorded would be required for a 6 hr. flight if glycogen alone was available. Possibly the aphids from natural infestations contained, and used, greater amounts of glycogen than those from culture, and the metabolic rates and contributions of glycogen were greater than

those calculated. The available evidence, however, suggests that, at least after the first hour, fat provides most of the energy for flight, i.e. *c.* 90% of the energy for a tethered flight of 6 hr.; the corresponding value for the locust *Schistocerca gregaria* during a flight of 5 hr. is 80–85% (Weis-Fogh, 1952).

Most of the metabolism during flight is in the flight muscles. The thorax constituted between 30·5 and 38·3% of the live body weight of the 24 hr. old insects (mean $= 35{\cdot}1\%$, $n = 9$) and the flight muscles occupied between 34·5–39·2% of the total volume of the thorax (mean $= 37{\cdot}0\%$, $n = 5$). Assuming that the density of the muscle tissue was similar to that of the remaining thoracic tissues, the flight muscles constituted *c.* 13% of the live body weight (cf. 18% in *Drosophila repleta*, Chadwick & Gilmour, 1940, and 13% in *D. melanogaster*, Hocking, 1953).

From this value, the approximate rates of metabolism of the muscles during flight in Expts. 1–5 were calculated; these varied between 400 and 500 cal./g. muscle/hr. Assuming that the proportion of water in the muscles was the same as that in the body as a whole (mean $= 68\%$), the mean rate of fuel consumption was 17% of the dry muscle weight/hr.

Flight capacity in relation to initial fat content

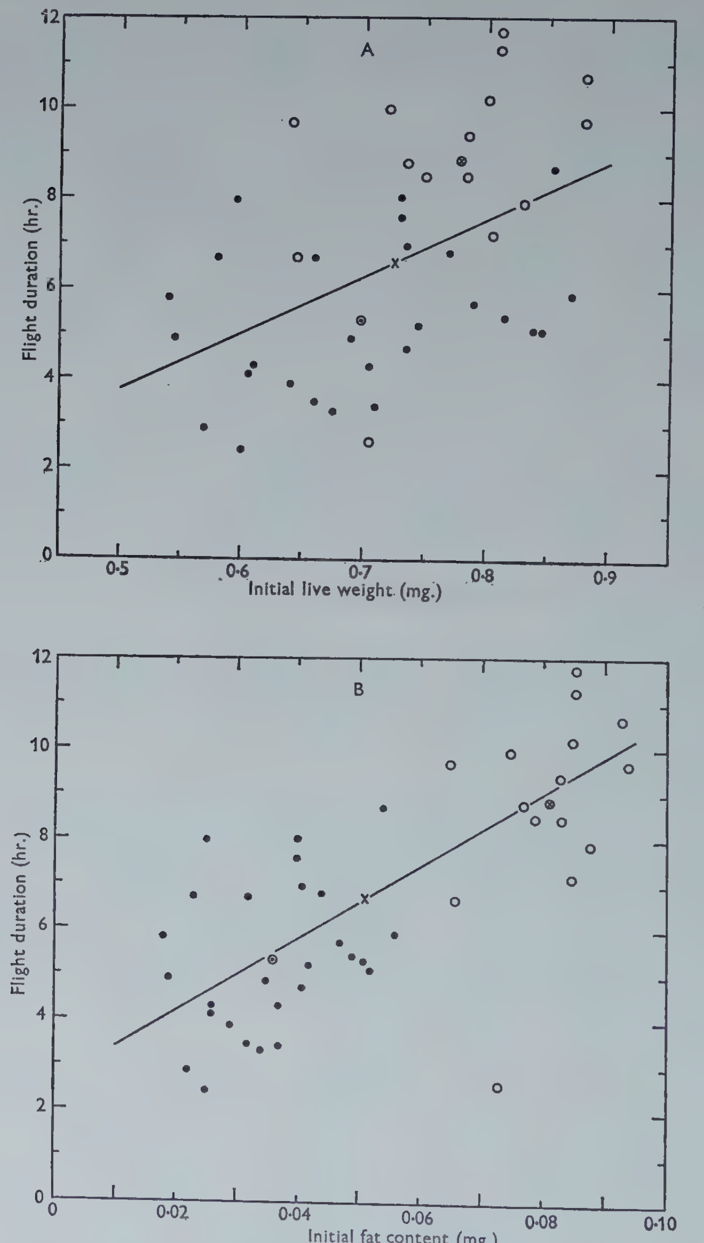
Two experiments (10 and 11) were made on flight capacity using aphids from the same sources as those used in Expts. 1 and 4 respectively, i.e. broad beans in culture and in the field. Aphids were weighed individually before flight and their initial fat contents estimated by interpolation of the appropriate control graph in Text-fig. 2; the values are only approximate for the regressions in Text-fig. 2 are based on mean, not individual, weights.

The mean flight duration was 5·3 hr. in Expt. 10 (culture aphids) and 8·9 hr. in Expt. 11 (field aphids). Text-fig. 6A, B show the durations plotted against initial live weight and fat content respectively. A slight correlation was found between duration and fat content in Expt. 10 ($r = +0{\cdot}324$; $P = 0{\cdot}10$), but none between duration and fat content in Expt. 11, nor between duration and initial live weight ($P > 0{\cdot}10$). The combined data, however, give highly significant correlations between duration and live weight ($r = +0{\cdot}490$; $P = 0{\cdot}001$) and between duration and fat content ($r = +0{\cdot}784$; $P = 0{\cdot}001$). When flight duration is considered in relation to initial live weight, the results from Expt. 10 (aphids with low fat contents) lie mainly below the common regression line, and those from Expt. 11 (aphids with high fat contents) above. When duration is plotted against initial fat content, however, a regression continuous from group to group is obtained.

The available evidence therefore suggests that flight capacity is limited by initial fat content; had other processes, e.g. neural fatigue, accumulation of waste metabolites, water loss (see Cockbain, 1961), etc., constituted limiting factors to flight, high correlation between flight duration and initial fat content in aphids from two sources would have been unlikely.

It may be calculated from the data in Table 3 that after a flight of 5·3 hr. aphids from culture would have used a mean of 51% of their initial fat, and after a flight of 8·9 hr. those from broad bean in the field would have used 56%. Thus in both experiments the mean proportion of initial fat used during flight was about the same, but in neither was exhaustion associated with entire depletion of fat. Some of the remaining 'fat' (ether extract) will not be fuel reserve, however, and the rest, in parts of the body

remote from the flight muscles (see next section), may not have been mobilized quickly enough to support wing movement (cf. mobilization of glycogen in *Drosophila*, Wigglesworth, 1949).



Text-fig. 6. Flight capacity at 25–26° C. in relation to (A) initial body weight ($b = +12{\cdot}67$) and (B) initial fat content ($b = +75{\cdot}13$). ●, Culture aphids; ⊙, mean for culture aphids; O, field aphids; ⊗, mean for field aphids; ×, common mean.

Distribution of the flight reserves

General anatomy of an alate aphid is shown in Pl. 1 A, and histology of the thoracic fat body in Pl. 1 B.

(*a*) *Distribution of fat.* Most of the fat lies in the fat-body cells of the thorax and abdomen. In the thorax the fat body surrounds all the organs; in the abdomen it is mainly restricted by the presence of embryos to the lateral and posterior regions. Typically the fat occurs as globules within the cytoplasmic network of the cells, but in unflown insects the staining of fat in many cells is so intense that the globular nature is masked. Most cells in the thorax are free from fat after prolonged flight to exhaustion (cf. Pls. 1 C, D, 2 E, F) but small deposits persist in the abdomen.

Fat also occurs between the fibrils of the indirect flight muscles, but no discrete droplets were detected. The muscle fat becomes less during flight, but the muscles stain for fat even when the insect has flown to apparent exhaustion (cf. Pl. 2 E, F).

(*b*) *Distribution of glycogen.* Glycogen was sparsely distributed and was principally in the fat body. Glycogen occurs in fixed material as small flakes, mainly around the periphery of the fat-body cells. It also occurs along the surface of the muscle fibres but none was detected within or between the fibrils. Glycogen was barely detectable in thorax or abdomen of insects that had flown for long periods (cf. Pl. 2 G, H).

SUMMARY

1. Fat contents (ether extracts) of unflown 24 hr. old alatae of *Aphis fabae* Scop., from different host plants, range from 3–12% of the live weight and 9–33% of the dry weight. Glycogen contents of alatae reared in culture range from 0·5–1% of the live weight and 1·7–3·4% of the dry weight.

2. Both fat and glycogen are consumed during tethered flight. Glycogen is used during early flight and fat is the principal fuel after the first hour, when it is consumed at a mean rate of 0·005 mg./aphid/hr. and provides about 90% of the energy for a 6 hr. flight. The amounts of glycogen in laboratory-reared aphids alone could not maintain flight for more than $\frac{3}{4}$ hr.

3. Metabolic rates during tethered flight range from 52 to 66 cal./g. live wt./hr. or 11–14 ml. O_2/g./hr. The flight muscles constitute about 13% of the live body weight of 24 hr. old aphids, and, attributing most of the metabolism during flight to these muscles, their metabolic rates range from 400 to 500 cal./g./hr.

4. Flight capacity of 24 hr. old aphids at 25–26° C. is directly related to initial fat content and varies between 3 and 8 hr. in aphids from culture (mean of 4% fat by live weight) and between 7 and 12 hr. in aphids from the field (10% fat). Flight fatigue occurs before all the fat reserves are used; possibly fat stored in parts of the body remote from the flight muscles cannot be mobilized rapidly enough to support continuous flight.

5. Fat and glycogen reserves occur mainly in the fat-body cells of the thorax and abdomen; fat also occurs between the fibrils of the indirect flight muscles and glycogen along the surface of the fibres. Flight-exhausted insects have little or no fat in the thorax, but small deposits remain in the abdomen; little glycogen can be detected in culture aphids flown to exhaustion.

It is a pleasure to express my thanks to Dr C. G. Johnson for his supervision and helpful criticism of this work, and to Mr L. R. Taylor and Dr K. Mellanby for reading the manuscript. My thanks are due also to Mr C. I. Carter for technical assistance; to Mr J. H. A. Dunwoody for the statistical analyses in Tables 2 and 3; and to Mr F. D. Cowland for taking the photomicrographs.

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EXPLANATION OF PLATES

PLATE 1

A. Sagittal section of one-day-old alate *Aphis fabae*. *c*, cauda; *dlm*, dorsal longitudinal flight muscle; *dvm*, dorso-ventral muscle; *e*, embryos; *f*, fat body; *m*, mid-gut; *t*, thoracic ganglion. PAS (after saliva treatment) and Mayer's haemalum, ×75.

B. Section of thoracic fat body of 6-day-old aphid showing cytoplasmic network, nuclei and fat vacuoles within the cells. Heidenhain's iron-haematoxylin, ×470.

C. One-day-old unflown aphid. Section of thorax beneath meso-scutellum stained for fat. Streaming appearance of the fat is a sectioning artifact. Sudan Black B and carmalum, ×470.

D. As for C, but flown for 5½ hr.

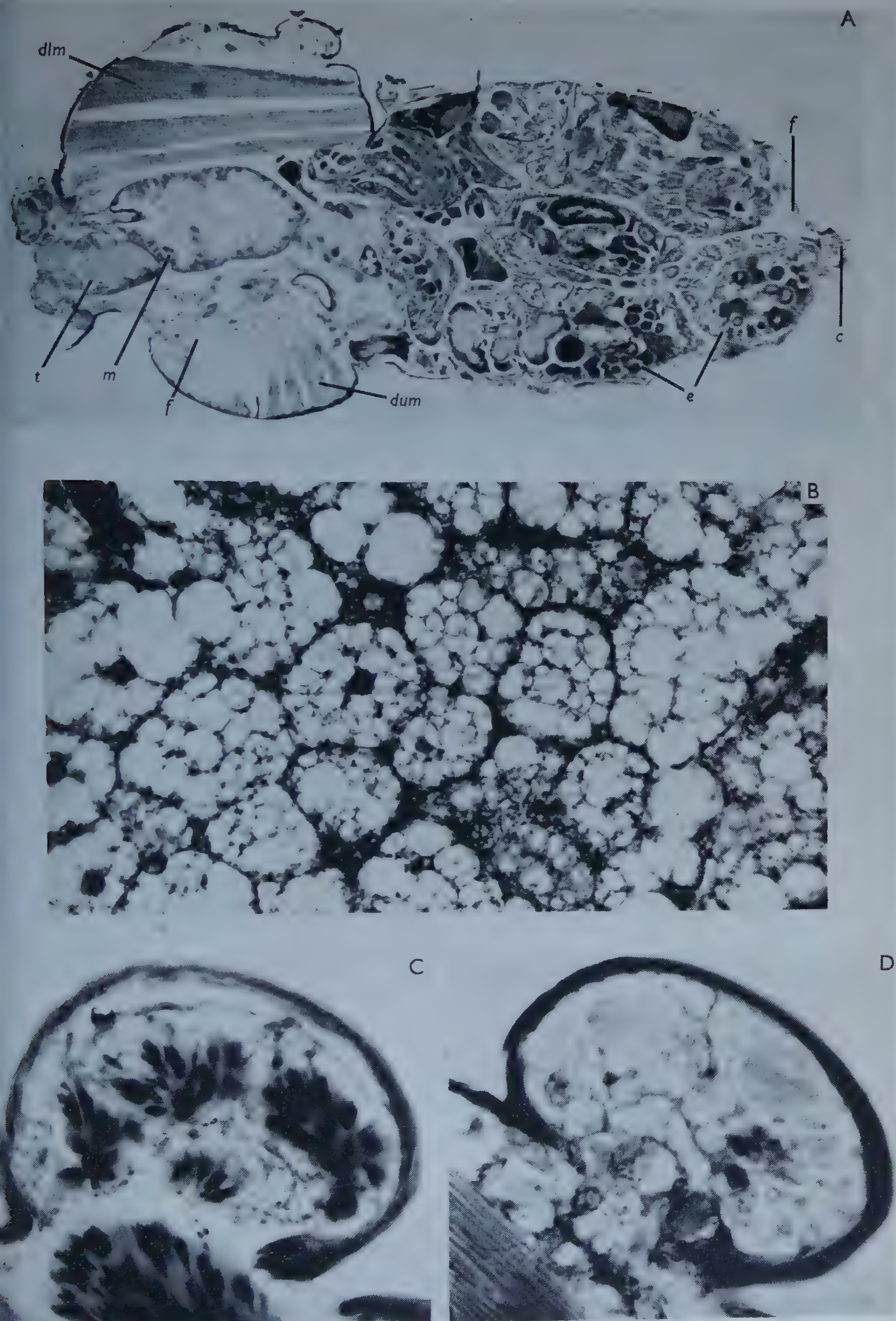
PLATE 2

E. One-day-old unflown aphid. Section of fat body and dorso-ventral muscle stained for fat. Sudan Black B and carmalum, ×170.

F. As for E, but flown for 8 hr.

G. One-day-old unflown aphid. Section of cauda stained for glycogen (*g*). PAS, ×470.

H. As for G, but flown for 12 hr.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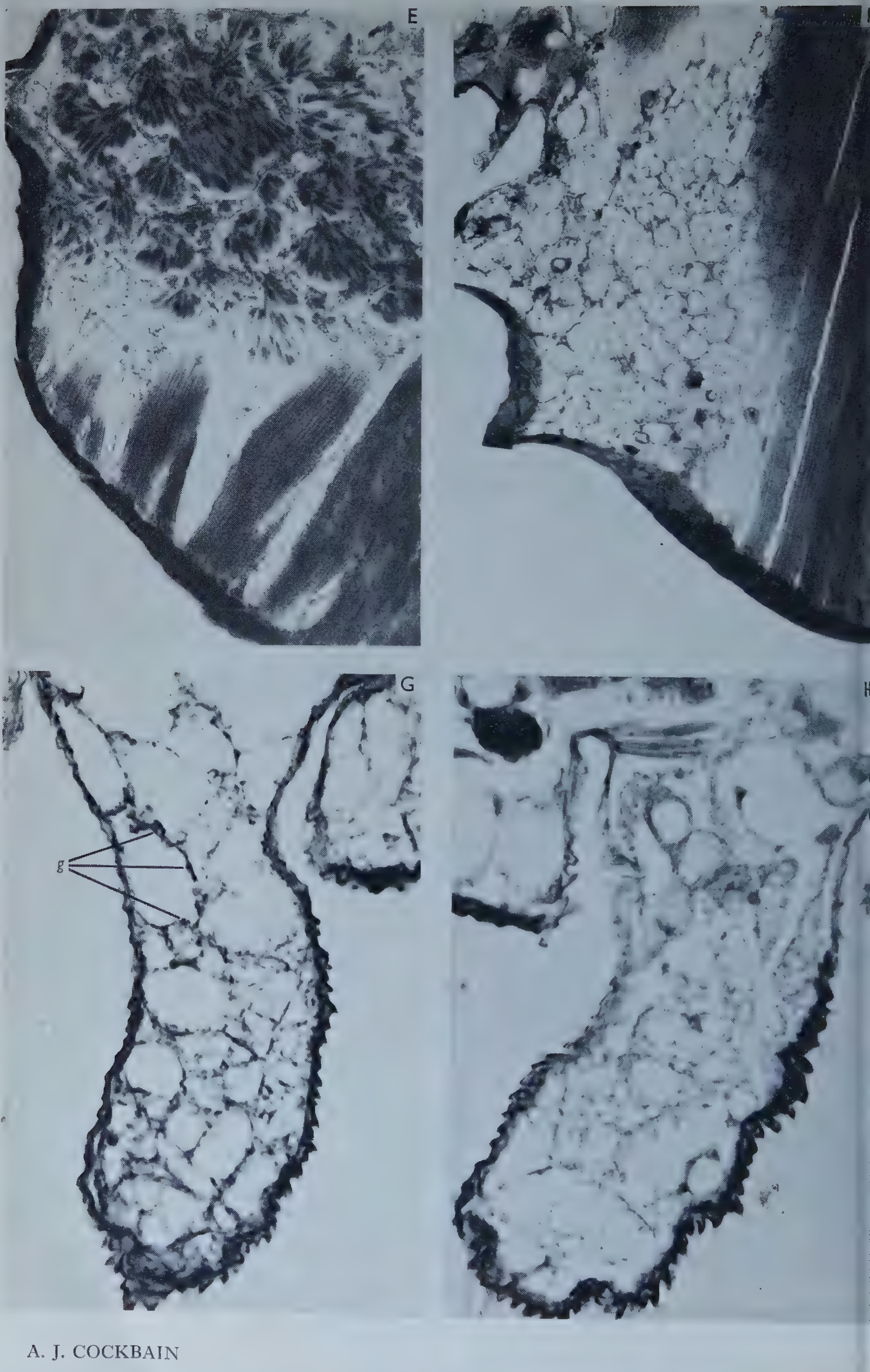

A. J. COCKBAIN

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WATER RELATIONSHIPS OF *APHIS FABAE* SCOP. DURING TETHERED FLIGHT

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(*Received* 12 *August* 1960)

INTRODUCTION

In a study of fuel used by *Aphis fabae* during tethered flight (Cockbain, 1961 *a*) results were obtained on the water balance of these insects during flight, and form the basis of this paper.

MATERIALS AND METHODS

Materials and methods are described fully in the previous paper. Water contents were estimated by subtracting insect dry weight from live weight; pre-flight water contents of flown aphids were interpolated from graphs relating water content to live weight in unflown control insects.

RESULTS

Water content before flight

Water content in 24 hr. old unflown alatae was directly proportional to total live weight (Fig. 1). The amount of water varied from 64 to 73 % live weight, values which were well within the range found in other insects (Buxton, 1932) and are only slightly lower than those recorded for alatae of *Macrosiphum pisi*, i.e. 74 % (Schaefer, 1938). The highest proportions of water were in aphids reared in the laboratory on broad beans, the lowest in those from a natural infestation of a field bean; variations between batches from the same host were small (Table 1).

Variations in the proportions of water in the aphids from different hosts were mainly associated with differences in the proportions of fat; those aphids with the highest percentage of fat had the lowest percentage of water (Fig. 2). Thus the hydration of fatless dry matter in the aphids from the different hosts was very similar (Table 1).

Water loss during flight

Table 2 gives mean live weights and water contents of batches of aphids before and after flight; pre-flight water contents were interpolated from the appropriate regression in Fig. 1 (mean water content/aphid).

Decreases in water content during flight were recorded in all batches. Fig. 3 (graph A) shows mean decrements per aphid. More water was lost than is indicated by these decrements, however, for some water will have been produced by oxidation of the flight reserves. For the present purposes, decrements in live weight during flight were taken as representing the total amounts of water lost, for the principal fuel used is fat, which yields almost an equivalent weight of water on oxidation. Fig. 3 (graph B) shows mean decrements in live weight.

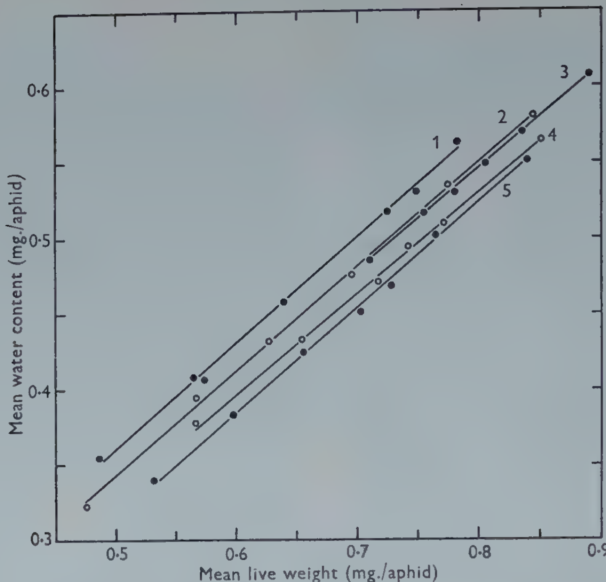

Fig. 1. Relationship between mean water content and live weight in unflown 24 hr. old alatae from different host plants. Numbers refer to experiments; regression coefficients given in Table 1.

Table 1. *Proportions of water in 24 hr. old unflown alatae*

Expt. no.	Host plant	Location	No. of batches × no. of aphids/batch	% water to live weight (min.–max.)	% water to fatless live weight (min.–max.)	Regression coefficient of water to live weight
1	Broad bean	Culture	7 × 10	71·0–72·5	74·3–76·3	+0·699
2*	Spindle	Field	6 × 10	68·1–69·5	74·6–75·7	+0·687
3	Dock	Field	6 × 10	68·2–68·6	73·4–73·8	+0·669
4	Broad bean	Field	6 × 10	65·6–66·5	73·1–74·3	+0·668
5	Field bean	Field	7 × 10	63·7–66·2	71·5–74·0	+0·701

* The alatae were migrantes, not alienicolae as in the other experiments.

The mean decrease in water content during 6 hr. was 0·039 mg./aphid, corresponding to 5·1 % initial body weight, but the mean total water loss was 0·072 mg./aphid, or 9·4 %. Fig. 3 shows that both rate of decrease in water content and rate of water loss were highest during the early period of flight, the rate of decrease in water content gradually falling as flight proceeded but the rate of total water loss remaining fairly constant after the first two hours. Thus a mean of 0·021 mg. water (3·0 % initial body weight) was lost per aphid during the first hour and 0·008 mg. (1·1 %) during the last hour. This apparently high rate of water loss (decrease body weight) during early flight was probably because aphids excreted more often (see below) and used more glycogen (Cockbain, 1961 *a*) during this period; it may also have been because moisture was lost from the surface of the cuticle.

Excretion and evaporation during flight

Water is lost during flight by excretion of honeydew droplets and by evaporation; excretory droplets are considerably smaller than those produced by aphids feeding on a host plant but nevertheless cause water loss; most of the evaporation undoubtedly

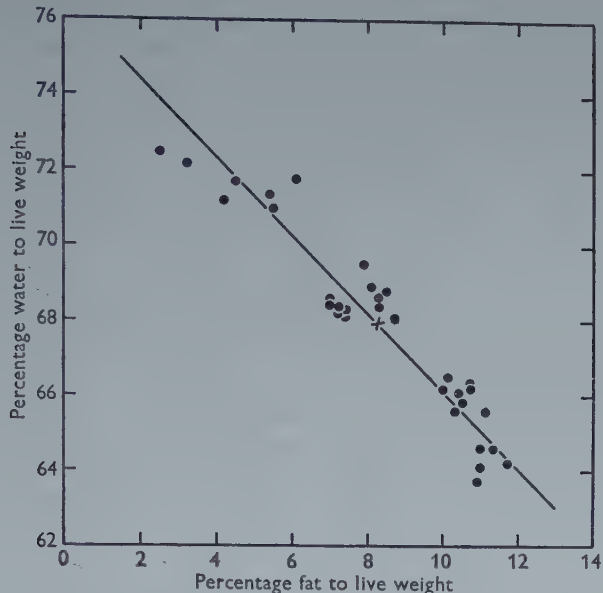

Fig. 2. Relationship between proportions of water and fat in unflown 24 hr. old alatae ($b = -0·971$). ×, mean value.

Table 2. *Live weights and water contents of batches of aphids before and after flight*

(Aphids flown at 25–26° C. and at different humidities.)

	Expt. 1 70±6% R.H.		Expt. 2 59±2% R.H.		Expt. 3 80±2% R.H.		Expt. 4 70±4% R.H.		Expt. 5 76±3% R.H.	
Aphid condition	Live weight (mg.)	Water content (mg.)	Live weight (mg.)	Water content (mg.)	Live weight (mg.)	Water content (mg.)	Live weight (mg.)	Water content (mg.)	Live weight (mg.)	Water content (mg.)
Before flight	5·91 (10)	4·24	6·115 (9)	4·20	6·705 (8)	4·58	5·19 (7)	3·43	7·35 (11)	4·76
After flight	5·76	4·12	5·89	4·00	6·53	4·465	4·98	3·305	7·21	4·63
Before flight	4·41 (8)	3·18	5·16 (8)	3·55	7·18 (9)	4·91	6·15 (8)	4·07	5·455 (8)	3·54
After flight	4·24	3·06	4·85	3·33	6·805	4·685	5·77	3·82	5·17	3·32
Before flight	6·43 (10)	4·61	5·25 (8)	3·62	7·425 (9)	5·08	6·235 (8)	4·12	5·375 (8)	3·48
After flight	6·00	4·33	4·775	3·275	6·88	4·755	5·72	3·785	4·97	3·24
Before flight	5·715 (8)	4·08	5·65 (7)	3·89	7·28 (9)	4·98	5·40 (7)	3·56	5·02 (7)	3·28
After flight	5·17	3·77	5·07	3·56	6·615	4·655	4·865	3·28	4·605	3·04

Values within parentheses refer to number of aphids per batch.

takes place through the spiracles (Mellanby, 1934), but some may be through the cuticle (Koidsumi, 1934; Church, 1960).

Excretion was not studied in the above experiments but was studied in three experiments in which culture aphids were flown for 4 hr. at 25–26° C. and at different humidities (41–75% R.H.); excretory droplets were collected on pH indicator paper. Table 3 gives the frequency of excretion and the mean diameter of the spots produced by the droplets on the paper (proportional to droplet size). Neither the mean number of droplets excreted nor the mean diameter of the excretory spots was correlated with relative humidity of the air, so humidity differences over this range do not affect excretion during flight.

Excretion was most frequent during early flight, but on the average only one droplet per aphid was produced during 4 hr. It may be inferred from extrapolation of the

data in Table 3 that little excretion would occur after 4 hr.; water loss during the last 2 hr. of flight in Expts. 1–5 was therefore almost entirely from evaporation, i.e. 0·008 mg./aphid/hr. Thus evaporation under these conditions probably accounts for *at least* 66 % of the water lost during a 6 hr. flight.

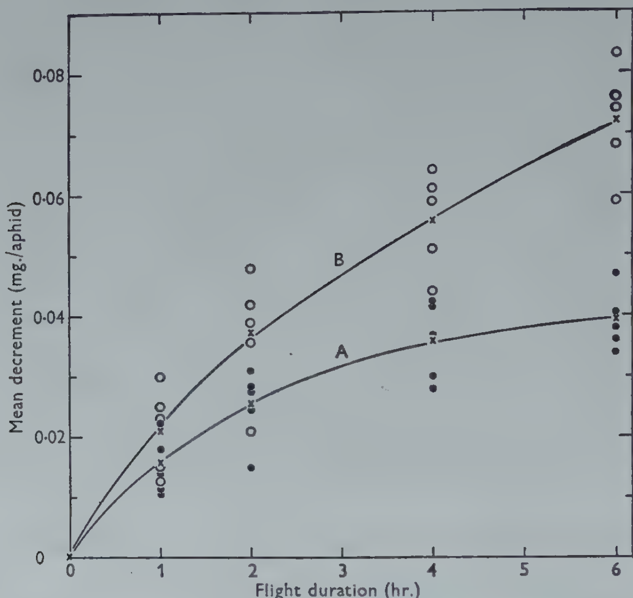

Fig. 3. Decrease in water content (A) and live weight (B) during flight at 25–26° C. and 57–82 % R.H. ×, mean values.

Table 3. *Frequency of excretion during flight at* 25–26° C. *and at different humidities, and sizes of excretory droplets as indicated by spot diameter on* pH *indicator paper*

Expt.	A		B		C	
Relative humidity (%) ...	70 ± 5		64 ± 4		47 ± 6	
Flight duration (hr.)	No. of aphids	Frequency of excretion (drops/aphid/hr.)	No. of aphids	Frequency of excretion (drops/aphid/hr.)	No. of aphids	Frequency of excretion (drops/aphid/hr.)
1	16	0·31	18	0·33	19	0·26
2	16	0·44	18	0·33	19	0·32
3	16	0·06	18	0·11	19	0·21
4	16	0	18	0·22	19	0·11
Mean no. droplets/aphid	—	0·81	—	0·99	—	0·90
Mean spot diameter (μ)	—	589 ± 122	—	535 ± 81	—	583 ± 103

There was a barely significant inverse relationship between the total amounts of water lost (% decrease body weight) during 4 and 6 hr. flights and the relative humidity of the air ($r = -0{\cdot}541$; $P = 0{\cdot}10$). On the average, an increase of 10 % R.H. was associated with a decrease in the amount of water lost of 0·8 % body weight, undoubtedly because humidity affected evaporation. The correlation might have been higher had the experiments been over a wider range of humidities.

Water content during flight

Although absolute water content decreases during flight, the proportion of water in the aphids tends to increase slightly (Fig. 4A), because the percentage of dry matter, i.e. fats and glycogen, used during flight is greater than that percentage of the water content which is lost. The mean increase (67·9–69·4 % body wt.) during 6 hr., however, was not significant ($t = 0{\cdot}83$; $P > 0{\cdot}10$).

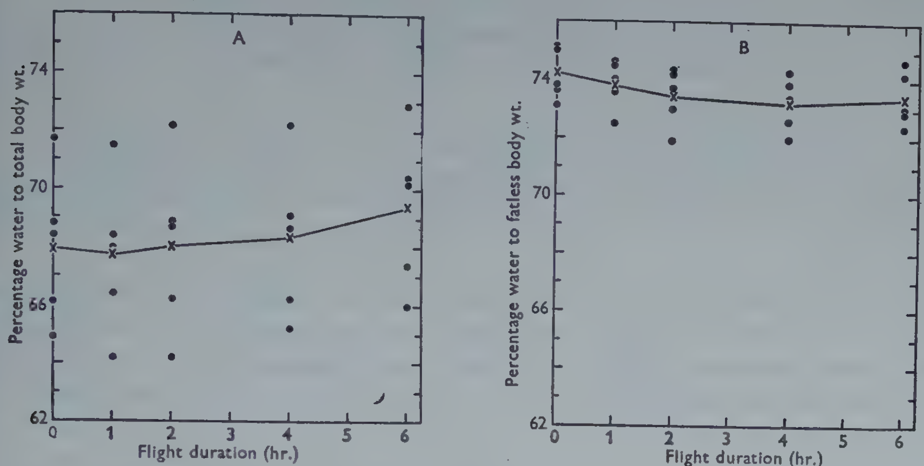

Fig. 4. Change in percentage water content during flight. A, Water content as percentage of total body weight; B, water content as percentage of fatless body weight. ×, mean values.

Hydration of fatless dry matter is probably important in connexion with the water balance, for fats are not associated with water in storage. The percentage of water to fatless body weight shows a tendency to fall during flight, reaching a minimum after 4 hr. (Fig. 4B). Even then the mean percentage has only fallen from 74·2 to 73·2 %; the difference between the means was not significant ($t = 1{\cdot}69$; $P > 0{\cdot}10$). During more prolonged flight the water balance would not alter appreciably, for although water would be lost at a mean of 0·008 mg./aphid/hr. (see above), about 0·005 mg./hr. would be gained by metabolism of fat reserves (see Cockbain, 1961 *a*). The results are similar to those obtained in flight studies on the locust *Schistocerca gregaria*, in which the hydration of non-fatty dry matter remains nearly constant or improves slightly during 5–9 hr. flight (Weis-Fogh, 1956).

CONCLUSIONS

Water was lost by excretion and evaporation during flight, but the proportion of water in the aphids and the hydration of lean dry matter remained almost constant. During more prolonged flight (> 6 hr.) the water balance would not be expected to change appreciably. It is therefore unlikely that water loss would limit the flight capacity of aphids flying tethered at 25–26° C. and 57–82 % R.H., or in more moist atmospheres; in these conditions, flight duration is probably limited by fuel content (Cockbain, 1961 *a*). Results given elsewhere show that the flight durations of culture aphids in humidities as low as 21–27 % R.H., at 25–26° C., do not differ significantly

from those of aphids flown in higher humidities (see table 1, Cockbain, 1961 *b*); water loss even in these dry conditions is not a limiting factor to flight. In drier atmospheres, however (saturated deficit > 23 mm. Hg), the amount of water lost by evaporation would probably greatly exceed that gained by metabolism; water loss might then be a limiting factor.

SUMMARY

1. Water content varies from 64 to 73 % of the total body weight and 72–76 % of the fatless body weight of 24 hr. old unflown alatae of *Aphis fabae*.

2. Water loss during flight may be attributed to evaporation and excretion. A mean of 0·07 mg. water is lost per aphid during a 6 hr. tethered flight at 25–26° C. and 57–82 % R.H., corresponding to *c.* 9 % body weight; at least 66 % of the loss (*c.* 1 % body weight/hr.) is by evaporation.

3. Excretion during flight is not affected by relative humidity differences over the range 41–75 % at 25–26° C., but the relative amounts of water lost during prolonged flight are inversely related to relative humidity, because of the effect of humidity on evaporation.

4. Proportion of water in the body does not change significantly during tethered flight. Mean percentage water to total body weight increases from *c.* 68–69 % during 6 hr.; mean percentage water to fatless body weight decreases from *c.* 74 to 73 %.

5. Water loss is evidently not a limiting factor to flight in atmospheres of saturation deficit less than *c.* 23 mm. Hg.

I wish to thank Dr C. G. Johnson, Mr L. R. Taylor and Dr K. Mellanby for criticizing the manuscript.

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With 2 text-figures
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VIABILITY AND FECUNDITY OF ALATE ALIENICOLAE OF *APHIS FABAE* SCOP. AFTER FLIGHTS TO EXHAUSTION

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(*Received* 12 *August* 1960)

INTRODUCTION

I have studied the effects of exhaustive flight on *Aphis fabae* Scop. to see if a long migratory flight is likely to affect the subsequent life and reproductive potential of aphids.

MATERIALS AND METHODS

Alate alienicolae of approximately uniform size were obtained from healthy colonies of the same clone maintained in the laboratory at $18 \pm 5°$ C. on broad beans (*Vicia faba* L., var. Claudia Aquadulce). They were collected within $\frac{1}{4}$ hr. of the final ecdysis and kept for 24 hr. on young beans in the dark at 20° C. to complete their teneral development and become ready for flight (Taylor, 1957).

The aphids were flown on pins (Cockbain, 1961) at 25–26° C., 21–84% R.H., and in an air stream of 1 m.p.h.

Experiments 1–5. Within each experiment there were two experimental (*a* and *b*) and two control (*c* and *d*) batches, 7–8 aphids per batch. Test aphids were flown to exhaustion and control aphids were flown for 15 min. in order to induce settling (see B. Johnson, 1958; Kennedy, 1958). After flight the aphids were kept in darkness on young beans, one batch per plant, at 20° C. and 45% R.H., and brought into light at the same temperature and humidity after 3 days, i.e. when they had lost the ability to fly because their flight muscles had autolysed (B. Johnson, 1957). Larvae produced by the aphids were counted and removed when the plants were changed every 2–3 days, or whenever an adult died.

Experiments 6–8. Test aphids were flown to exhaustion and then starved in glass tubes in darkness at 20° C. and 70% R.H.; unflown controls were starved under the same conditions.

RESULTS

Flight durations and subsequent behaviour

Behaviour during prolonged tethered flight and determination of the point of exhaustion have been described elsewhere (Cockbain, 1961). Most aphids flew continuously for long periods, requiring only occasional stimulation, until they became fatigued. Further flight may have been possible after a period of rest as in *Drosophila* (Wigglesworth, 1949), but it is doubtful whether the duration of flight could have been increased significantly. Flight durations in different experiments were consistent enough to give confidence in the method.

Table 1 gives mean, minimum and maximum durations. Flights of 3–9 hr. are

typical of tethered culture aphids in the temperatures and humidities stated above; variations are partly because the amounts of flight reserves differ (Cockbain, 1961). Expt. 8 was at a very low humidity (mean saturated deficit 23 mm. Hg), but the mean flight duration did not differ significantly from that of the other experiments; water loss by evaporation, related to the humidity of the air, is evidently not a limiting factor to flight under these conditions.

Table 1. *Flight durations at 25–26° C. of the 1-day-old aphids flown to exhaustion*

Expt. no.		1		2		3		4		5		6	7
R.H. (%)		70±3		67±4		67±3		71±3		67±4		67±4	79±5
Batch no.		*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	—	—
No. of aphids		8	8	8	7	8	8	7	7	8	8	14	16
Flight duration (hr.)	Mean	4·9	6·8	5·4	7·8	5·6	7·9	5·6	7·7	5·1	7·5	5·5	6·1
	Min.	3·3	5·5	3·8	6·9	4·4	7·3	4·7	6·5	4·5	6·3	2·5	2·6
	Max.	5·9	8·1	6·6	9·4	6·5	8·6	6·2	9·6	6·1	8·6	8·0	8·7

The exhausted aphids in Expts. 1–5 settled and began to feed within a few minutes of being placed on the plants. A few of the controls attempted to take-off after probing, but flight was prevented by placing the insects in darkness. None of the exhausted aphids was disturbed when exposed to light for 10–30 min. on the following day, but some controls immediately began to wander and flight had to be prevented again by darkness. An attempt was made to fly the exhausted aphids of two experiments (4–5) on the second day. Some would not fly and the others were difficult to start. Those which failed to fly could raise their wings but appeared unable to beat them; this is a sign of the beginning of flight-muscle autolysis and further flight may have been impossible. Table 2 shows mean, minimum and maximum durations of second flights, and the percentage of aphids which failed to fly. The corresponding controls, batches 4*c* and 4*d*, 5*c* and 5*d*, were flown with no difficulty for a further 15 min.

Table 2. *Flight durations on the second day of aphids previously flown to exhaustion*

Expt. no.		4		5	
Batch no.		*a*	*b*	*a*	*b*
% unable to fly		0	57	38	0
Flight duration (hr.)	Mean	3·1	1·0	1·7	2·0
	Min.	1·9	0	0	0·6
	Max.	4·3	3·7	4·3	3·3

Of the exhausted aphids in Expts. 6–8 (starved after flight) twenty-eight per cent were able to take-off and fly when exposed to direct sunlight on the following day. Most of them flew upwards towards the light, as is characteristic of first flight. The remaining exhausted aphids could not take-off, some being moribund or dead. Of the unflown controls eighty-eight per cent were able to take-off and fly on the second day.

Longevity after flight

Tethered flight to exhaustion did not affect the longevity of aphids allowed to settle and feed on a host plant after flight (Expts. 1–5). The mean adult life of each batch ranged from 24 to 36 days for exhausted aphids and 28–37 days for controls (Table 3). A maximum life of 41 days for individual aphids occurred with both exhausted and control insects. Within the same experiment, one control batch (3 *d*) lived significantly longer than an exhausted batch (3 *b*) ($t = 2{\cdot}37$; $P = 0{\cdot}05$–$0{\cdot}02$), and one exhausted batch (5 *a*) lived significantly longer than a control (5 *d*) ($t = 2{\cdot}39$; $P = 0{\cdot}05$–$0{\cdot}02$). Mean adult lives of 31·1 and 32·4 days for combined data for exhausted and control aphids respectively showed no significant difference ($t = 1{\cdot}03$; $P > 0{\cdot}10$).

Table 3. *Mean adult longevities of aphids feeding on young bean plants (Expts.* 1–5)

Exhausted aphids			Control aphids		
Batch no.	Mean longevity (days)	S.D. (±)	Batch no.	Mean longevity (days)	S.D. (±)
1 *a*	28·5*	8·9	1 *c*	34·6*	1·5
1 *b*	32·9	4·7	1 *d*	30·0	5·4
2 *a*	34·3	4·5	2 *c*	37·0	3·1
2 *b*	36·3	2·5	2 *d*	31·7	9·8
3 *a*	34·5	4·2	3 *c*	32·0	9·2
3 *b*	24·3†	11·8	3 *d*	35·3†	3·4
4 *a*	25·0**	11·0	4 *c*	34·0**	1·6
4 *b*	33·9	2·2	4 *d*	30·0	9·1
5 *a*	34·5††	1·3	5 *c*	31·1	6·0
5 *b*	26·9	8·2	5 *d*	28·3††	6·9
Mean	31·09	8·29	Mean	32·36	6·75

*, ** Differences significant at 10 % level; †, †† differences significant at 5 % level.

Fig. 1 shows survival curves for exhausted and control aphids in Expts. 1–5. The curves deviate slightly when all aphids are considered (1 A) but are almost identical when the comparison is restricted to aphids of uniform size, i.e. hind tibia length 1·125–1·250 mm. (80 % of the exhausted and 75 % of the control aphids in Expts. 1–5) (Fig. 1 B).

Aphids starved after flight exhaustion lived for a significantly shorter time than aphids starved without flight (Table 4). Mean survival time of flown aphids in Expts. 6–8 was 30·3 hr., i.e. they lived for *ca.* 24·5 hr. after flight; mean survival time of unflown aphids was 55·2 hr.; these means were significantly different ($t = 7{\cdot}68$; $P < 0{\cdot}001$). The unflown controls in Expt. 6 lived significantly longer than those in Expts. 7 and 8 ($t = 3{\cdot}48$ and $3{\cdot}54$ respectively; $P = 0{\cdot}01$–$0{\cdot}001$); this was associated with their greater size, as indicated by hind tibia length. There was a positive correlation between survival time and hind tibia length in the control aphids ($r = +0{\cdot}346$; $P = 0{\cdot}05$–$0{\cdot}02$), such that a difference of 0·1 mm. tibia length was associated with a difference of 13 hr. in length of life during starvation; no correlation was found

between survival time and hind tibia length in the exhausted aphids ($r = -0{\cdot}086$; $P > 0{\cdot}10$), nor between length of life after flight and flight duration in these aphids ($r = -0{\cdot}119$; $P > 0{\cdot}10$).

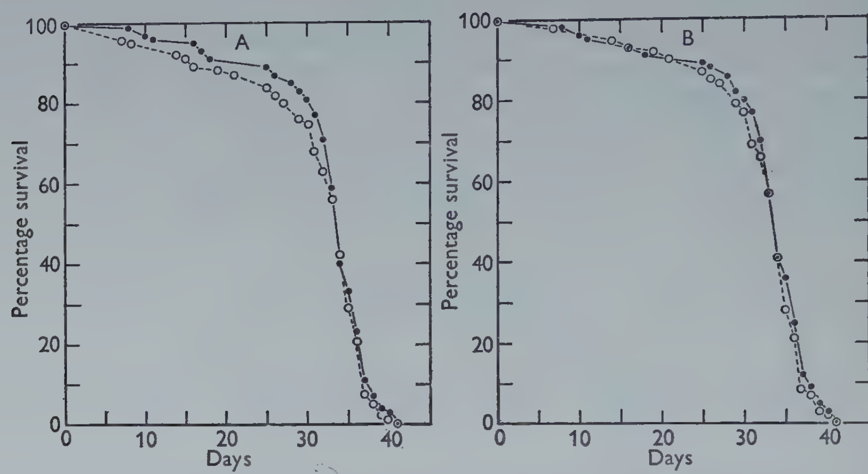

Fig. 1. Mean percentage survival of aphids feeding on young bean plants (Expts. 1–5). A, all aphids; B, aphids with hind tibia length 1·125–1·250 mm.; ○- - - -, exhausted aphids; ●——, control aphids.

Reproduction after flight

Flight to exhaustion had no effect on rate of larviposition or on total number of larvae deposited by aphids allowed to feed after flight. The mean accumulative totals of larvae born to exhausted and control aphids in Expts. 1–5 were similar (Fig. 2A, B). The highest rate of larviposition was during the 24 hr. following flight in both exhausted and control aphids, with means of 11·4 and 11·0 larvae/aphid respectively. The rate then decreased to a mean of 2 larvae/aphid/day after 4 days and increased to 6 larvae/aphid/ day after about 12–14 days. A decrease occurred towards the end of the reproductive period which varied from 22 to 29 and 22 to 30 days in the exhausted and control batches respectively.

The reproductive capacities of aphids which completed their reproductive life were about the same in all batches, the combined data giving a mean of 84·2 larvae per adult for both exhausted and control insects (Table 4).

Table 4. *Mean survival times of aphids starved at* 20° C. *and* 70% R.H. (*Expts.* 6–8)

Exhausted aphids					Control aphids				
Expt. no.	Survival times* (hr.)	S.D. (±)	Hind tibia length (mm.)	S.D. (±)	Expt. no.	Survival time* (hr.)	S.D. (±)	Hind tibia length (mm.)	S (
6	32·9	13·9	1·137	0·035	6	67·0	10·7	1·160	0·
7	29·0	17·0	1·148	0·041	7	47·6	17·5	1·120	0·
8	28·8	12·4	1·085	0·041	8	50·9	10·2	1·103	0·
Mean	30·3	15·0	1·128	0·047	Mean	55·2	16·3	1·130	0·

* Includes flight times.

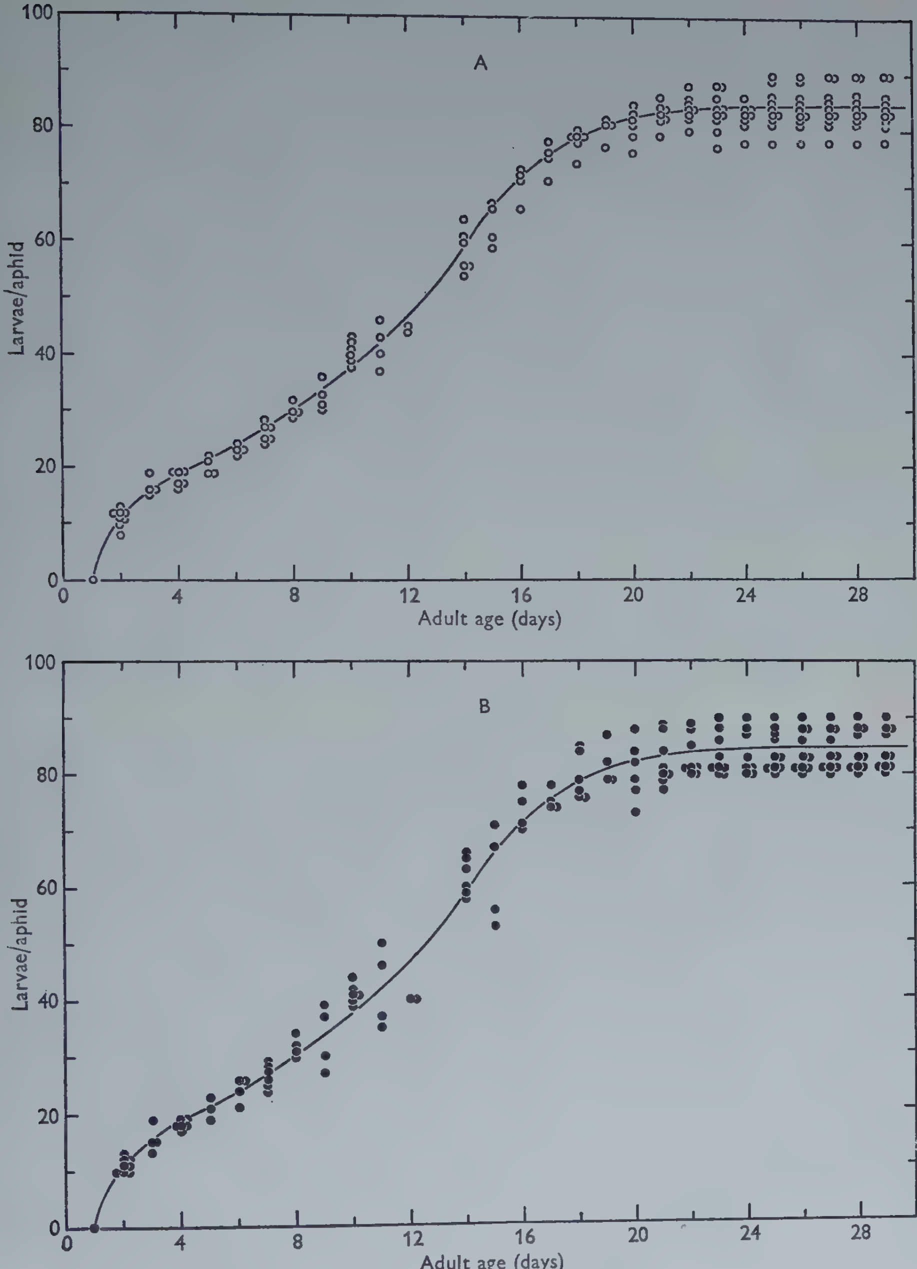

Fig. 2. Accumulative total number of larvae produced per aphid as a function of adult age; each point represents the mean of a batch. A, Exhausted aphids; B, control aphids.

Mortality of nymphs born of flight-exhausted aphids

Data in the preceding section show that exhaustive flight had no adverse effects on the development and birth of larvae carried during flight, or on further embryogenesis. Nor was the viability of these larvae affected, for pre-adult mortality of larvae born

during the first week after flight in Expt. 1 was similar in both exhausted and control batches. Of 437 and 458 larvae born to the exhausted and control aphids respectively, 9 (2·1 %) and 8 (1·8 %) failed to complete development.

Table 5. *Number of larvae deposited by the exhausted and control aphids that survived the reproductive period* (*Expts.* 1–5)

Exhausted aphids				Control aphids			
Batch no.	Reproductive period (days)	% reaching post-reproductive period	Mean no. larvae/aphid	Batch no.	Reproductive period (days)	% reaching post-reproductive period	Mean no. larvae/aphid
1*a*	22	87·5	84·2	1*c*	24	100·0	89·9
1*b*	22	87·5	85·8	1*d*	22	87·5	80·6
2*a*	27	87·5	90·4	2*c*	30	100·0	87·7
2*b*	29	100·0	90·4	2*d*	24	83·3	83·2
3*a*	24	100·0	84·7	3*c*	23	87·5	81·4
3*b*	22	62·5	83·3	3*d*	22	100·0	80·7
4*a*	23	66·7	80·9	4*c*	22	100·0	88·4
4*b*	22	100·0	82·6	4*d*	26	71·4	87·9
5*a*	22	100·0	82·2	5*c*	23	87·5	82·7
5*b*	23	62·5	77·9	5*d*	22	75·0	79·7
Mean	23·6	85·4	84·2	Mean	23·8	89·2	84·2

CONCLUSIONS

C. G. Johnson (1957) and Taylor (1958) showed that the average duration of flight of aphids in the field is probably of the order of 1–3 hr.; similar times for aphids flying freely in a flight chamber were recorded by Kennedy and Booth (1956). In the present study the aphids were flown tethered to pins for 3–9 hr., to apparent exhaustion of the available flight reserves; such flights, providing the aphids later settled and fed on a host plant, had no adverse effects on their subsequent life. The rate at which flight reserves are consumed may differ in free and tethered flight; 1 hr. of tethered flight may not be equivalent in terms of energy expenditure to 1 hr. of natural flight. Nevertheless, it appears unlikely that prolonged flight in the field lowers the viability (see also Taylor, 1960) or reproductive potential of aphids; provided that an aphid alights on a suitable host, length of life and fecundity are unlikely to be affected by flight duration.

Most natural migrants that experience prolonged flight and fail to alight on a host plant, or other plant from which they can derive some nourishment, probably live for only a short time. Results indicate that a few may be able to take-off on the following day; the first few seconds of flight is then similar in some respects to first flight, i.e. upwards towards light (C. G. Johnson, 1955); these aphids would have another chance to alight on a suitable host.

Those aphids that were flown to exhaustion and then allowed to settle and feed on young bean plants in darkness made no attempt to fly when exposed to light on the second day; some could not do so even when stimulated, possibly because of the early onset of flight-muscle autolysis. It is reasonable to infer that a natural migrant of *A. fabae*, alighting on a suitable host after an exhaustive flight, would not fly again.

SUMMARY

1. Laboratory-reared 24 hr.-old alate alienicolae of *A. fabae* were flown for variable periods, but all to apparent exhaustion; their subsequent longevity and fecundity on broad beans, or their survival times during starvation, were compared with controls.

2. Adult longevity, reproductive rate and capacity, and nymph viability were similar in exhausted and control aphids that settled on host plants after flight. Mean adult life was 31 days in exhausted aphids and 32 days in the controls. Both exhausted and control aphids produced an average of 84 larvae per adult.

3. The only major difference noted between exhausted and control aphids that fed after flight was a reluctance, or inability, of exhausted aphids to fly on the following day.

4. Exhausted aphids starved after flight lived for a significantly shorter time (mean of 30 hr.) than control aphids starved without flight (55 hr.). 28% of the exhausted and 88% of the control aphids could take-off on the next day.

5. The results indicate that long migratory flights are unlikely to affect the reproductive potential of aphids, and that alate alienicolae of *A. fabae*, having settled on a suitable host after an exhaustive flight, are unlikely to fly again.

I am most grafeful to Mr L. R. Taylor for his advice and encouragement during the course of the work, and to Dr C. G. Johnson and Dr K. Mellanby for reading and criticizing the manuscript.

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THE ORIENTATION OF *LITTORINA* SPECIES TO POLARIZED LIGHT

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INTRODUCTION

Plane polarized light has been shown to influence the orientation of numerous invertebrates, for example the decapod crustacean *Eupagurus bernhardus* ((Kerz, 1950), mysids (Bainbridge & Waterman, 1957, 1958), species of Cladocera (Baylor & Smith, 1953) and numerous insects (Vowles, 1950, 1954; Carthy, 1951, 1957, 1958; Stephens, Fingerman & Brown, 1953; Frisch, 1950; Stockhammer, 1956; Wellington, Sullivan & Green, 1951; Wellington, 1953, 1955; Kalmus, 1958, 1959, to name but a few). It has also been shown that the receptors of the xiphosuran *Limulus* can detect changes in the plane of vibration of polarized light (Waterman, 1950, 1954*a*, *b*). More recently the ability to orientate to polarized light has been demonstrated in the marine gastropod *Littorina littoralis* (L.) by Burdon-Jones & Charles (1958*a*, *b*) and in *Nassa obsoleta* by Baylor (1959).

The investigations on *Littorina littoralis* have now been extended to other British species of the Littorinae, and their behaviour gives some indication of the means by which the plane of vibration of the light is detected by these molluscs.

APPARATUS AND PROCEDURE

Fig. 1 shows diagrammatically the essential details of the apparatus used. The light originated from a 500 W. spotlamp (*A*), passing successively through a condenser (*B*), a water-bath heat filter (*C*), a ground glass screen (*D*) serving as a depolarizer, and finally through a Polaroid linear polarizer (*E*). The latter was fitted into a rotatable housing so that the plane of vibration of the light could be altered at will. This arrangement provided an illumination of 50 ft.c. at the centre of the horizontal surface over which the animals were allowed to crawl.

The crawling surface for the trials conducted with the molluscs crawling in air consisted of a circular Perspex plate *c.* 40 cm. in diameter, the upper surface of which had been roughened with fine grade carborundum paste to facilitate wetting. A black non-reflecting grid ruled in centimetre squares was placed beneath the Perspex plate, the lines being clearly visible through the wetted surface.

For the trials using animals fully immersed in sea water, the Perspex plate was replaced by a circular glass trough (*F*) *c.* 32 cm. in diameter. A matt black screen (*G*) fitted within the trough minimized any reflexion of light from the walls. To reduce further any reflexions from the wall of the experimental cell and to prevent the regular

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pattern of light and dark areas produced when polarized light struck the meniscus at the margin of the water surface, a black annular diaphragm (*H*) was fitted to shield the walls and meniscus from direct illumination. The whole apparatus was surrounded by a tall cylindrical screen (*I*), blackened on its inner surface, and a wide annular mask (*J*) fitted horizontally around the Polaroid holder to prevent any spurious reflexions from above. All experiments were conducted in a dark room.

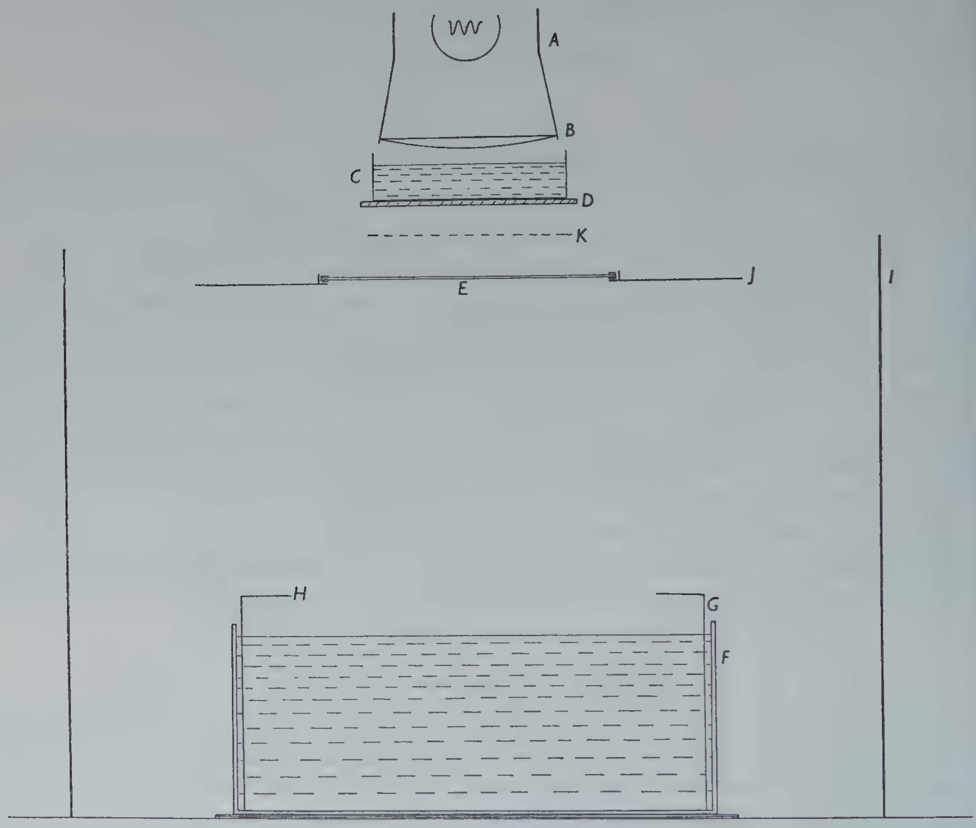

Fig. 1. Diagram of the apparatus used to test the responses to polarized light of freely moving *L. littoralis* (L.) and *L. littorea* (L.) immersed in sea water. *A*, 500 W. tungsten filament spot-lamp; *B*, condenser; *C*, water-bath heat filter; *D*, ground glass screen depolarizer; *E*, polaroid linear polarizer fitted into a rotatable housing; *F*, circular glass trough containing sea water, beneath which was placed a matt black grid ruled in centimetre squares; *G*, cylindrical matt black screen; *H*, matt black annular diaphragm; *I*, cylindrical matt black screen; *J*, matt black annular mask; *K*, position of colour filter when employed.

In a typical trial a winkle was placed facing in any direction at the centre of the crawling surface and allowed to crawl about freely until it reached the periphery. The winkles left a readily visible mucus trail on the Perspex plate surface. With the aid of the centimetre grid, facsimiles of their tracks were plotted on to a large sheet of graph paper after the completion of each trial.

The mucus trails were erased after each trial lest they should interfere with subsequent trials.

When *Littorina littoralis* (L.) and *L. littorea* (L.) were crawling immersed in sea water in the large glass trough their tracks were recorded while the winkles were in motion. Since the trials were conducted in a dark room stray reflexions from the observer were minimal and did not interfere with the illumination of the trough in any way. The results obtained did not indicate any directional bias that might have been induced by the presence of the observer.

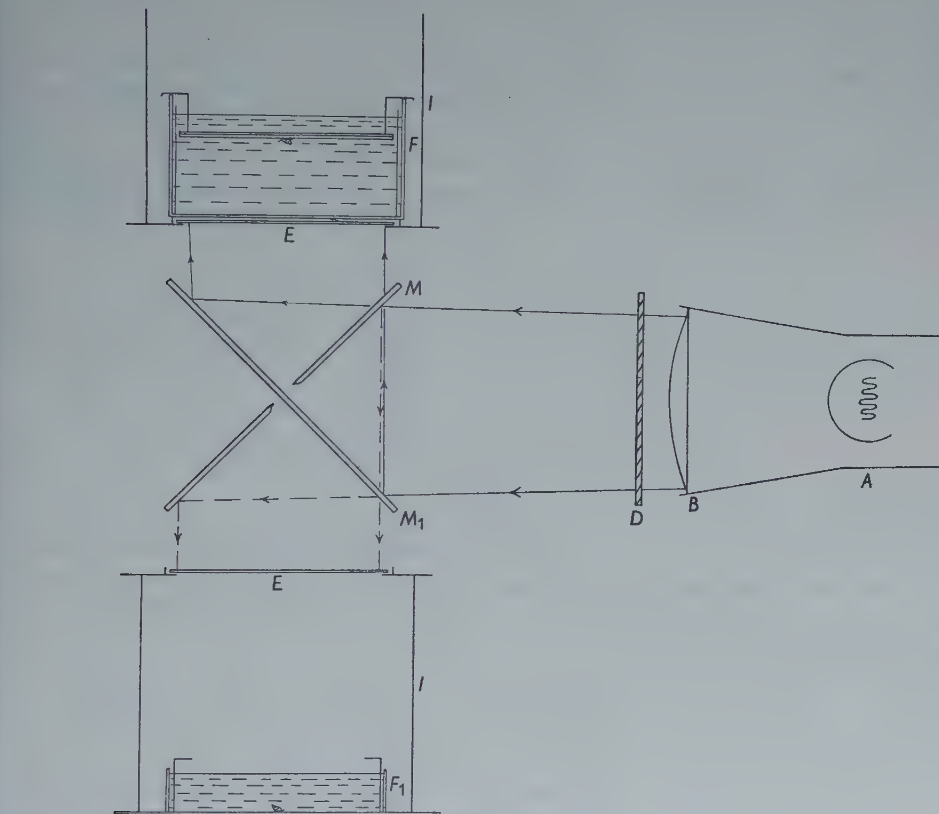

Fig. 2. Diagram of the apparatus used to test the responses to polarized light of freely moving *L. saxatilis* (L.) and *L. neritoides* (L.) whilst crawling upright or inverted immersed in sea water. *A*, 500 W. tungsten filament spotlamp; *B*, condenser; *D*, ground glass screen depolarizer; *E*, polaroid linear polarizer; *F*, experimental chamber with a clear glass bottom, filled with sea water and containing the inverted Perspex plate crawling surface; F_1, experimental chamber filled with sea water with a Perspex plate crawling surface as the base of the chamber; *I*, matt black cylindrical screens; *M*, position of the plane mirror for the trials with the winkles crawling upright; M_1, position of the plane mirror for the trials with the winkles crawling upside down.

Since the common periwinkle *L. littorea* (L.) may be either photonegative or photo-ositive (Newell, 1958*a*, *b*), the type of response was determined with ordinary light efore making any trials with polarized light. This was done by removing the Polaroid rom the apparatus and placing a white reflecting surface against one half of the black nner circular screen to form a cylindrical arena with one half white and the other lack. With central overhead illumination photopositive winkles crawled towards ne white half of the arena and photonegative ones towards the black half.

The small size of *L. neritoides* (L.) and the need to study its response to polarized light while crawling upside down led to the apparatus being modified as shown in Fig. 2. The light beam was reflected upwards or downwards into the experimental chamber (*F*) by means of the plane mirror (*M*), with the Polaroid (*E*) interposed between the mirror and the experimental chamber. For the trials with *L. neritoides* in the inverted position the winkles were first allowed to attach themselves normally to the Perspex plate which was then quickly turned over and immersed in the dish of sea water.

The arrangement of screening to prevent spurious reflexions was essentially similar to that already described. The possibility of light being reflected from the observer did not arise, since mucus trails were used to record the tracks. To predetermine their sign of response to light the same technique as described for *L. littorea* was used. The modified apparatus was also used for the trials with *L. saxatilis* (L.).

ANALYSIS OF RESULTS

The reference axis employed was a predetermined horizontal direction in space related to the room in which the experiments were carried out, and assigned the bearing 0–180°. Angles were measured in a clockwise direction from 0 to 360°.

Experiments on Littorina littoralis (*L.*)

In preliminary experiments with *L. littoralis* (Burdon-Jones & Charles, 1958*a*) three experimental conditions were applied:

(*a*) The plane of vibration (*e* vector) of the light was kept parallel with the reference axis, until the animal had traversed a standard distance from the starting-point when the plane of vibration was momentarily turned through 90° and returned to its original position.

(*b*) The plane of vibration was rotated through 90° from the reference axis after the animal had traversed the standard distance, and left in its new position.

(*c*) The same as for (*b*) but with no Polaroid in the rotatable holder.

In these experiments the angle subtended by the end-point of the track and the reference axis was measured. The number of individuals whose tracks terminated less than 30° from the reference axis is given in column 2 of Tables 1 and 2. A large proportion of the trials fell into this category when the plane of vibration was parallel to the reference axis. Hence there is a definite tendency for *L. littoralis* to orientate by crawling parallel to the plane of vibration whether immersed in sea water or in air. When the Polaroid was rotated through 90° a consequent turn of 90° was induced in the mollusc, as can be seen from the higher figures in column 4 than in column 2 of Tables 1 and 2, for the second part of group B. Application of the χ^2 test showed the results to be highly different from random expectation. In applying this test, it was assumed that one third of those that reached the periphery would be expected in each of columns 2–4.

The response of *L. littoralis* to polarized light of different wavelengths was investigated by using a series of Chance colour filters (*K*) placed between the depolarizer and the Polaroid. For these trials and subsequent trials with polarized light on the other species, the tracks were analysed in a slightly different manner and in more detail.

Table 1. Littorina littoralis (*L.*) *immersed in sea water. Angles given are deviations from the original plane of vibration of the light,* 0–180°

Treatment	0–30° 150–180° 180–210° 330–360°	30–60° 120–150° 210–240° 300–330°	60–90° 90–120° 240–270° 270–300°	No. circling	Total no. tested	χ^2	Value of P for two degrees of freedom
up A. Plane of vibration stant along 0–180° reference s. First half of track before ation of polaroid through 90° l return to reference axis	37	6	1	6	50	46·41	$< 0·005$
nd half of track after rotation polaroid through 90° and return reference axis	40	3	1	6	50	65·75	$< 0·005$
up B. Plane of vibration ated through 90°. First half rack before rotation through	35	9	0	6	50	45·03	$< 0·005$
nd half of track after rotation ough 90°	7	18	19	6	50	6·04	$< 0·05$, $> 0·025$
up C. In non-polarized light	10	17	10	13	50	2·65	$< 0·5$, $> 0·25$

Table 2. Littorina littoralis (*L.*) *in air. Angles given are deviations from the original plane of vibration of the light,* 0–180°

Treatment	0–30° 150–180° 180–210° 330–360°	30–60° 120–150° 210–240° 300–330°	60–90° 90–120° 240–270° 270–300°	No. circling	Total no. tested	χ^2	Value of P for two degrees of freedom
up A. Plane of vibration stant along 0–180° reference s	81	7	2	0	90	130·47	$< 0·005$
up B. Plane of vibration ated through 90°. First half rack before rotation through	83	6	1	0	90	138·97	$< 0·005$
nd half of track after rotation ough 90°	13	26	51	0	90	24·87	$< 0·005$
up C. In non-polarized light	24	30	25	11	90	0·78	$< 0·75$, $> 0·5$

The resultant angle made by each centimetre of track was measured. Those parts of the track which were approximately parallel to the reference axis were then estimated by counting the number of centimetres where the track lay within the sectors 0–30°, 150–180°, 180–210° and 330–360° on either side of the reference axis. They were expressed as a percentage of the total distance crawled. They were then compared with the percentage of the track which lay in the sectors 60–90°, 90–120°, 240–270° and 270–300° on either side of the axis at right angles to the reference axis. Table 3 is given as an example of this method of tabulating the results. It shows the responses of *L. littoralis* to polarized light within the range of wavelength 3500–6500 Å. The results were analysed statistically by applying Student's t test to ascertain whether the mean difference between the pairs of the percentages of the two equal angular groups was significantly greater than zero (Davies, 1949, t test difference method). [In conducting these experiments with polarized light of different wavelengths on *L. littoralis*,

Table 3. Littorina littoralis (*L.*) *in air. Response to polarized light of wavelengths between* 3500 and 6500 Å. (*blue-green filter*)

Winkle no.	% distance crawled 0–30°, 150–180°, 180–210°, 330–360° from reference axis (*e* vector)	% distance crawled 60–90°, 90–120°, 240–270°, 270–300° from reference axis	Difference
1	52·0	16·0	+36·0
2	63·6	0·0	+63·6
3	52·3	20·4	+31·9
4	90·5	0·0	+90·5
5	43·6	32·8	+10·8
6	80·0	0·0	+80·0
7	40·6	37·5	+ 3·1
8	4·0	16·0	−12·0
9	13·6	40·9	−27·3
10	58·8	12·9	+45·9
11	28·6	25·0	+ 3·6
12	100·0	0·0	+100·0
13	53·8	14·0	+39·8
14	68·0	16·0	+52·0
15	69·2	15·4	+53·8
16	85·0	5·0	+80·0
17	53·1	20·4	+32·7
18	28·2	33·4	− 5·2
19	43·5	26·1	+17·4
20	12·5	62·5	−50·0
		Mean difference	+32·33

Standard error (S.E.) associated with the mean difference = ± 8·97 %. Value of t = 3·6. Degrees of freedom ϕ = 19. Value of P is < 0·001 by the single-tail t test.

which had already been found to orientate parallel to the *e* vector (Burdon-Jones & Charles, 1958*a*, *b*), we were only interested in whether the tracks parallel to the plane of vibration exceeded those at right angles to it. As a difference in a particular sense was expected a single-tailed probability curve was employed for Student's t test. This was done by halving the probability value P obtained from the ordinary tables for t.]

This method was employed for all subsequent trials on *Littorina* species. Table 4 summarizes the results of the trials of *L. littoralis* to polarized light of different wavelengths. When light was transmitted through the blue-green filter (3500 and 6500 Å., first two sections of Table 4), *L. littoralis* showed a significant tendency to align itself parallel with the plane of vibration of the light. The t test gave P < 0·01 in both sets of experiments. However, trials with the other three colour filters decreased the efficiency of orientation, between the distance traversed parallel to the plane of vibration (column 2) and the distance crawled at right angles to this plane (column 3).

No attempt was made to equate the light energies for each wavelength in these comparative trials, but the relative intensities were of similar order. The relative intensities of the light to that of the standard apparatus without colour filter (50 ft.c. at the centre of the crawling surface) was obtained from the transmission curves for

Table 4. Littorina littoralis (*L.*) *in air. Response to polarized light of different wavelengths*

Illumination	Average % distance crawled within a 30° sector from: The reference axis (*e* vector)	The plane at right angles to the reference axis	Mean difference	S.E. associated with the mean difference	Total no. of animals tested	t	ϕ	P
lue-green filter between 3500 and 6500 Å.	52·04	19·21	+32·33	± 8·97	20	3·60	19	< 0·005*
lue-green filter between 3500 and 6500 Å.	48·56	20·54	+28·02	± 7·60	20	3·68	19	< 0·005*
reen filter between 4300 and 6500 Å.	36·58	33·60	+ 2·98	±10·24	20	0·29	19	< 0·4,* > 0·35
range filter from 5200 Å. into infra-red	39·51	32·65	+ 6·86	± 9·01	20	0·76	19	< 0·25,* > 0·2
ed filter from 5650 Å. into infra-red	40·81	29·77	+11·04	± 9·08	20	1·21	19	< 0·15,* > 0·1

* Single-tail t test.

the filters used and the spectrum of the tungsten filament light source. These were as follows: blue-green, 11 ft.c.; green, 11 ft.c.; orange, 21 ft.c.; and red, 10·5 ft.c. at the centre of the crawling surface.

Thus the results showed that *L. littoralis* was capable of orientating to plane polarized light between wavelengths of 3500 and 6500 Å. (blue-green filter) and of relatively low intensity (11 ft.c.), but orientation was less pronounced at other wavelengths despite the fact that these were of similar orders of intensity and, in the case of the orange filter, considerably higher (21 ft.c.).

Experiments on Littorina saxatilis (*L.*)

The interesting observations of Fraenkel (1927) on the reversal of phototaxis from photonegative to photopositive in *L. neritoides* while crawling inverted under sea water prompted a similar investigation (using the apparatus shown in Fig. 2) with *L. saxatilis*, since in some localities the habitats of the two species are almost identical. No analogous phenomena were apparent. *L. saxatilis* gave the same response as *L. littoralis* and orientated parallel to the plane of vibration whether upright or inverted. Both species were invariably photonegative when tested in the black and white arena. The results of the trials with *L. saxatilis* are summarized in section A of Table 5.

The results of trials of both the upright and inverted individuals showed that a greater proportion of the distance crawled by *L. saxatilis* tended to be parallel with the plane of vibration (column 2) than at right angles to it (column 3). The results were significant at the 0·05 level for the inverted trials. No doubt a higher degree of significance would have been obtained for both sets of trials if the number of trials had been larger.

Table 5. *Responses of* Littorina *species to polarized light*

Series and conditions of trials	Average % distance crawled within a range of 30° from: The reference axis (*e* vector)	The plane at right angles to the reference axis	Mean difference	S.E. associated with the mean difference	Total no. of animals tested	t	ϕ	P
A. *L. saxatilis* crawling upright, immersed in sea water, photonegative	44·71	25·49	+19·22	±11·19	22	1·72	21	0·1
Crawling upside down, immersed in sea water, photonegative	45·87	26·37	+19·5	± 9·35	20	2·08	19	< 0·1 < 0·05
B. *L. littorea* crawling upright in air, photonegative	40·12	29·59	+10·53	± 8·26	30	1·27	29	< 0·3, > 0·2
Crawling upright, immersed in sea water, photonegative	64·64	13·20	+51·44	±14·17	5	3·63	4	< 0·05 > 0·02
Crawling upright in air, photopositive	21·00	42·67	−21·67	± 6·78	30	3·19	29	< 0·01 > 0·00
Crawling upright, immersed in sea water, photopositive	17·30	56·68	−39·38	± 5·82	29	6·77	28	< 0·00
C. *L. neritoides* crawling upright immersed in sea water, photonegative	53·19	14·57	+38·62	±10·65	12	3·63	11	< 0·01 > 0·00
Crawling upright, immersed in sea water, photopositive	39·84	28·67	+11·17	±16·78	4	0·66	3	< 0·6, > 0·5
Crawling inverted, immersed in sea water, photonegative	47·49	21·87	+25·62	±15·24	10	1·68	9	< 0·2, > 0·1
Crawling inverted, immersed in sea water, photopositive	20·88	55·66	−34·78	±10·78	6	3·22	5	< 0·05 > 0·02

Experiments on Littorina littorea (*L.*)

In contrast to *L. littoralis* and *L. saxatilis*, *L. littorea* is irregular in its response to light (Newell, 1958*b*), being sometimes photopositive and sometimes photonegative, but rarely or never indifferent. At the time these experiments were conducted on *L. littorea*, the air temperatures were in the region of 18–20° C. and the animals behaved very sluggishly except when immersed in sea water. Therefore a series of trials was carried out with the animals under water as well as in air. The results are summarized in section B of Table 5. They show that both in air and under water photonegative *L. littorea* behave in a similar manner to *L. littoralis*, the percentage distance crawled within a 30° sector from the plane of vibration (column 2) being higher than that within a 30° sector of the plane at right angles (column 3). The t test gave values of $P < 0{\cdot}3$, $> 0{\cdot}2$ for the trials in air and $P < 0{\cdot}05$, $> 0{\cdot}02$ for the trials under water. Photopositive *L. littorea*, both in air and under sea water, orientated just as efficiently but at right angles to the plane of vibration, the distance travelled within a 30° sector of this plane (column 3 of section B) being much greater than the distance traversed within a 30° range either side of the plane of vibration (column 2). The values of P determined by the t test for these photopositive periwinkles were highly significant for both sets of trials ($P < 0{\cdot}01$, $> 0{\cdot}001$; and $P < 0{\cdot}001$).

Newell (1958*b*) noted that on horizontal surfaces *L. littorea* tended to loop back and forth on its tracks when orientating to the sun's position. Similar looping movements were noted in many cases when this species orientated itself in relation to the plane of vibration of polarized light.

Experiments on Littorina neritoides (*L.*)

The reversal of the light responses of *L. neritoides* reported by Fraenkel (1927) suggested that its reaction to polarized light should be tested when crawling normally and when crawling upside down. At water temperatures in the region of 20–22° C. their responses to light were erratic, but in sea water cooled to any temperature between 10 and 12° C. their behaviour was more consistent.

The results are summarized in section C of Table 4. Like *L. littorea*, photonegative *L. neritoides* crawled parallel to the plane of vibration when they were upright or when inverted, the greater proportion of the distance travelled being within a 30° sector of the plane of vibration (column 2). Like photopositive *L. littorea*, photopositive *L. neritoides* crawled at right angles to the plane of vibration, the greater percentage of the distance traversed being within a 30° sector each side of a plane at right angles to the *e* vector (column 3) than in the corresponding sectors parallel to this plane. The results of trials with the animal upside down were fairly conclusive (P values $< 0·2$, $> 0·1$; and $P < 0·05$) as were the trials with photonegative winkles crawling in the upright position ($P < 0·01$, $> 0·001$). The negative results obtained with photopositive winkles when crawling upright were probably the outcome of insufficient trials.

POSITION AND STRUCTURE OF THE EYES

The eyes in the family Littorinae are relatively small structures (about 240μ along the long axis of the optic cup in an adult *L. littoralis*) embedded in the outer sides of the tentacles at about one-third the distance from their bases. In Fig. 3 the angular settings of the eye in relation to the tentacle and longitudinal axis of the mollusc are shown. The optic cup is set obliquely with the lens aperture facing upwards, outwards and slightly anterior. Directly above and extending over the outer side of the eye the external layers of the tentacle are transparent and afford the eye a wide field of vision dorsolaterally.

It is apparent from Fig. 3 that with the optical axis of the eye subtending an angle of *c.* 40° with the vertical axis of the winkle (Fig. 3C) and *c.* 45° with the longitudinal axis (Fig. 3A) considerable depression of the head and tentacles is required before the eyes can receive any light reflected from the substratum close at hand or from the foot of the winkle, despite the slight forward tilt of the optic cup from the vertical axis (Fig. 3B).

In shape the optic cup is ellipsoid with a slight flattening frontally in the region of the lens aperture. The region between the lens and the retinal cup is filled with a clear gelatinous substance. A layer of dense black pigment surrounds the retina and encloses the whole of the optic cup with the exception of the lens aperture, effectively shielding the retina from any light except that admitted by this aperture.

The lens was examined under a polarizing microscope and by sectioning. It was

seen to consist of a series of concentric spherical laminae which showed no rotation of the plane of vibration of polarized light when viewed through crossed nicols. The eyes of the other winkles studied were similar in structure.

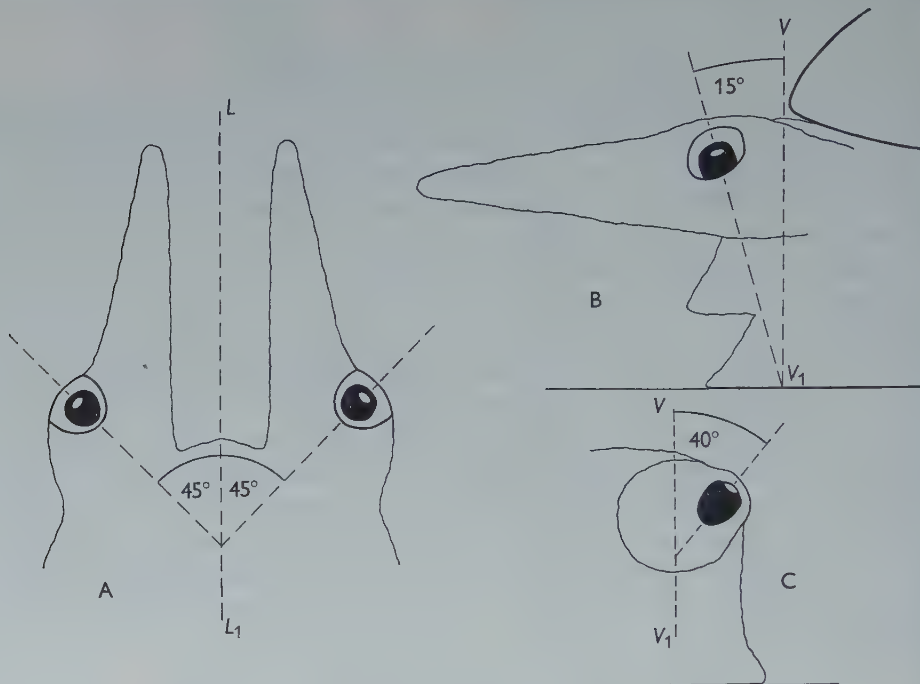

Fig. 3. The angular settings of the optic cup of *L. littoralis* (L.) in relation to the tentacle and the longitudinal and vertical axes of the mollusc. A, Dorsal view of the head and tentacles showing the optic cups subtending an angle of 45° with the longitudinal body axis L, L_1. B, Lateral view of the left tentacle, showing the optic cup tilted forwards and subtending an angle of *c.* 15° with the vertical body axis V, V_1. C, Anterior view of a diagrammatic section of the base of the left tentacle showing the optic cup subtending an angle of 40° to the vertical body axis V, V_1.

DISCUSSION

From the results for the four species of winkle investigated, it is reasonable to correlate the response to plane polarized light with the simple response to light and shade. Photonegative winkles invariably orientated parallel to the plane of vibration, and photopositive winkles at right angles to it. These facts, together with the absence of birefringence in the lens structure, point to a simple non-birefringent mechanism for the detection of the plane of vibration of the light. Such a mechanism was put forward by Stephens *et al.* (1953) for the orientation of *Drosophila* to polarized light, and was also favoured by Baylor & Smith (1953) for the orientation mechanism of *Daphnia* and other Cladocera. It was also listed among the possible analysing mechanisms by Waterman (1953, 1955), Stockhammer (1956), Bainbridge & Waterman (1957), Kalmus (1959) and favoured by Newell (1958*b*) as the probable means of orientation for *L. littorea*.

This mechanism is based on Fresnel's laws of refraction of polarized light. These are concerned with the percentage of light refracted at the interphase of two non-

birefringent media, expressed as a function of the angle of incidence, the indices of refraction of the media and the plane of vibration.

With reference to Fig. 4A and B, let I_0 represent the intensity of a beam of plane polarized light that is incident at an angle i at the interphase between two media whose indices of refraction are n and n_1 respectively. Let r represent the angle of refraction and I the intensity of the reflected beam. The proportion of light reflected, provided there is no loss by scatter, will be I/I_0 and the proportion refracted will be $1-(I/I_0)$.

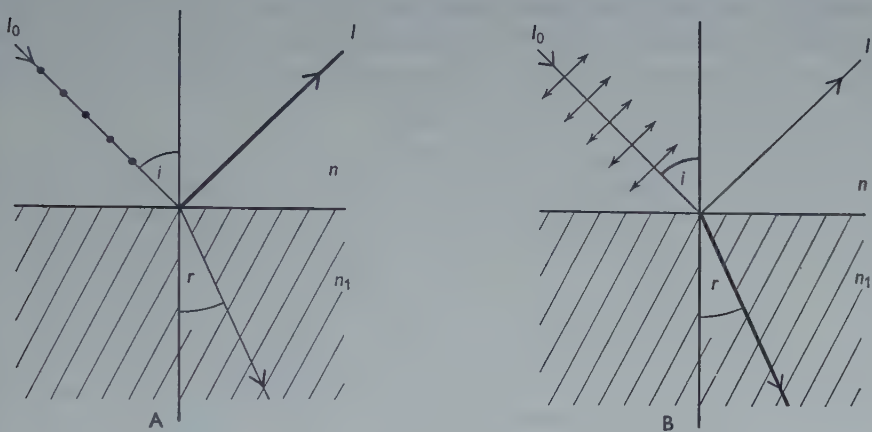

Fig. 4. Diagrams showing the reflexion and refraction of a ray of plane polarized light incident at the interphase of two media of differing refractive indices. I_0, intensity of the incident ray of polarized light; I, intensity of the reflected ray of polarized light; i, angle of incidence of the incident ray; r, angle of refraction of the refracted ray; n, n_1, media of differing refractive indices. A, When the plane of vibration of the incident ray is perpendicular to the plane defined by the incident and reflected rays, more light is reflected and less light refracted into the medium n_1. B, When the plane of vibration of the incident ray is in the same plane as that defined by the incident and reflected rays, less light is reflected and more light refracted into the medium n_1.

When the plane of vibration of the incident light is perpendicular to the plane defined by the incident and reflected rays (Fig. 4A), Fresnel showed that

$$\frac{I}{I_{0\,\text{perp.}}} = \frac{\sin^2(i-r)}{\sin^2(i+r)}.$$

When the plane of vibration of the incident rays is in the same plane as that defined by the incident and reflected rays (Fig. 4B) Fresnel showed that the intensity I of the reflected beam is given by

$$\frac{I}{I_{0\,\text{para.}}} = \frac{\tan^2(i-r)}{\tan^2(i+r)}.$$

Hence if we compare the amount of light reflected under the two conditions, that is with the plane of vibration perpendicular to and parallel to the plane of the incident and reflected rays, we have

$$\frac{I}{I_{0\,\text{perp.}}}\bigg/\frac{I}{I_{0\,\text{para.}}} = \frac{\cos^2(i-r)}{\cos^2(i+r)} = \frac{1+\sin 2i\sin 2r}{1-\sin 2i\sin 2r}.$$

Since both i and r lie between $0°$ and $90°$, $\sin 2i \sin 2r$ is always positive and less than unity, and the value of $[I/I_0]_{perp.}$ exceeds $[I/I_0]_{para.}$; conversely, the proportion of light refracted will, for any angle of incidence except $0°$ and $90°$, always be greater when the plane of vibration of the polarized light is in the plane defined by the incident and reflected rays.

The angular settings of the flattened region of the lens aperture and optic cup in the Littorinae are in the ideal positions for these phenomena to occur (Fig. 3).

When the head of the mollusc is parallel to the plane of vibration (e vector) of polarized light coming from above the animal, as shown in Fig. 5A, the plane of vibration is perpendicular to the plane of the rays incident and reflected from the eye surface. By Fresnel's laws the minimum amount of light is then refracted into the eyes.

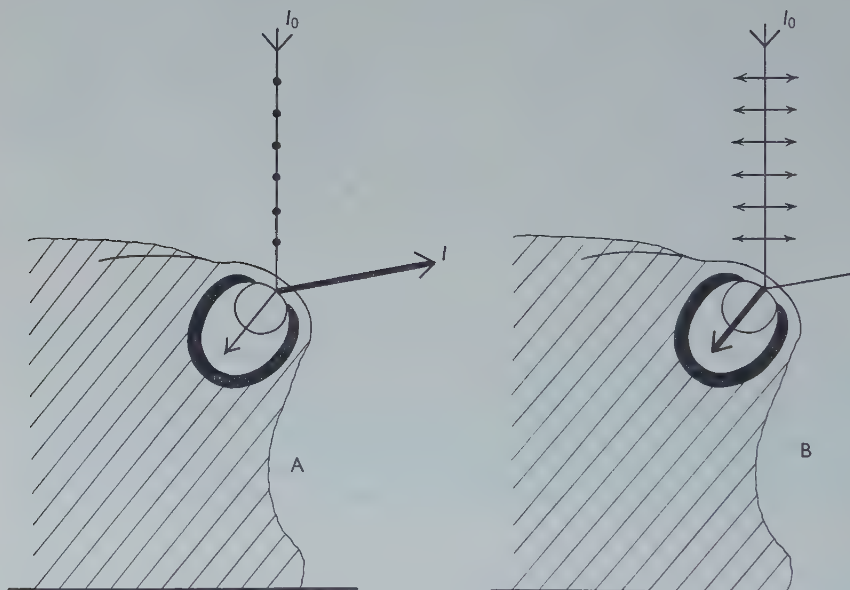

Fig. 5. Anterior views of transverse sections of the left side of a winkle head, showing the left optic cup. I_0, Vertical ray of plane polarized light incident upon the lens aperture; I, reflected ray of light. A, When the winkle is crawling parallel with the plane of vibration of the incident light, less light is refracted on to the retina. B, When the winkle is crawling at right angles to the plane of vibration of the incident light, more light is refracted on to the retina.

Conversely when the head is at right angles to the plane of vibration (Fig. 5B), the maximum amount of light is refracted into the eyes. Consequently, as the winkle turns its head to align itself parallel with the plane of vibration, less light is refracted on to the retina, simulating the effect of moving towards or into the shade.

As a result of the exploratory movements of the head a photonegative animal will orientate its body and move in a direction which results in the minimum amount of light being refracted into its eyes, i.e. along the plane of vibration. Conversely if photopositive, the animal will move at right angles to the plane of vibration so that the maximum amount of light will enter its eyes. Although exploratory movements of the tentacle are mainly executed by the distal portion, some movement of the

basal portion bearing the eye does occur. This, together with swinging movements of the head, will enable the eyes to move through a considerable angle and so detect changes in the amount of light received by the retina.

The concentric laminae of the lens of the eye in these animals would tend to accentuate any reflexion/refraction effect. Increasing the number of refracting layers will increase the number of times that the relative differences between the intensities of the reflected and refracted light are altered, since at each phase boundary the Fresnel effect will operate in proportion to the values of n_1/n_2, the refractive indices of the two phases. This would increase the effect of light and shade experienced by the winkle when it moved the plane of the eye surface in relation to the *e* vector of the polarized beam.

SUMMARY

1. Four British species of *Littorina*, viz. *L. littoralis* (L.), *L. saxatilis* (L.), *L. littorea* (L.) and *L. neritoides* (L.), responded when crawling on horizontal surfaces in air and in sea water to plane polarized light incident from above. Photonegative winkles crawled parallel to the plane of vibration (*e* vector) and photopositive molluscs at right angles to the plane of vibration.

2. The investigations with *L. neritoides* confirmed the observations of Fraenkel (1927) on the reversal of phototaxis from photonegative to photopositive when this winkle was crawling in the inverted position immersed in sea water. This change of response was considerably influenced by the sea-water temperature, the optimum temperature being within the range from 10° to 12° C.

3. Examination of the eye failed to reveal birefringent structures. It is suggested that the mechanism of analysing plane polarized light is a simple reflexion/refraction phenomena based on Fresnel's laws of refraction of polarized light, the minimum amount of light being refracted into the eye when the animal is crawling parallel to the plane of vibration.

I wish to thank Dr C. Burdon-Jones for his help and advice at all times in the supervision of this research, and Dr D. J. Crisp for many helpful suggestions, for his consideration of the statistical problems involved and for the concluding formula derived from Fresnel's laws.

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THE MECHANISM OF ORIENTATION OF FREELY MOVING *LITTORINA LITTORALIS* (L.) TO POLARIZED LIGHT

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INTRODUCTION

Several workers have stressed the importance of the intensity gradients produced where plane polarized light is reflected from the substrate or scattered by suspended particles in connexion with the orientation of animals to polarized light (Baylor & Smith, 1953, 1957, 1958; Baylor & Kennedy, 1958; Bainbridge & Waterman, 1958; Baylor, 1959; Kalmus, 1958, 1959). In a previous paper (Charles, 1961) the orientation of *Littorina* species to plane polarized light was discussed in relation to an intraocular analyser. A simple reflexion/refraction mechanism based on Fresnel's laws of refraction was suggested, whereby the intensity of light refracted on to the retina varied with the directional orientation of the animal. It was shown that photonegative winkles tended to crawl parallel to the plane of vibration and photopositive individuals at right angles to this plane.

This paper describes and discusses further experiments designed to test the validity of this theory, and to assess the relative importance of intraocular and extraocular perception of polarized light in the orientation mechanism of the Littorinidae.

INFLUENCE OF LIGHT INTENSITY CHANGES ON THE ORIENTATION TO PLANE POLARIZED LIGHT

The purpose of these trials was to vary the light intensity in accordance with the orientation of the mollusc in such a fashion that any intensity variation produced by a reflexion/refraction mechanism would be amplified or attenuated. *Littorina littoralis*, the species which gave the most consistent reaction to plane polarized light, was used in these experiments.

Apparatus and procedure

The apparatus was basically similar to that previously described (Charles, 1961), but modified by the insertion of a 'variac' transformer and a pointer mechanism controlling the lamp circuit. The pointer was positioned outside the experimental arena such that it could be aligned with the longitudinal body axis of the winkle when the mollusc was crawling freely over the roughened Perspex surface. It was also positioned in such a way as to ensure that there would be no interference from stray reflexions either from the pointer mechanism or its operator. The pointer was coupled to the 'variac' transformer by means of reduction gearing and a crank in such a manner that a quarter revolution of the pointer would raise the light intensity from

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a minimum to its maximum value, the succeeding quarter revolution would lower it to the minimum again, the next one would raise the intensity again, and so forth. This could be done in either direction of rotation.

Thus the pointer could be coupled with the 'variac' so that when the longitudinal body axis of the winkle was parallel with the plane of vibration the pointer aligned to it brought the light intensity to its maximum, namely, *c.* 50 ft.c. Conversely, as the winkle turned to a position at right angles to the plane of vibration and the pointer followed, the light intensity was reduced to a very low value of less than 1 ft.c.

Alternatively, by rotating the pointer through 90° in its coupling arrangement with the 'variac', it could be coupled in the opposite sense so that the light intensity, when the pointer was at right angles to the plane of vibration, was *c.* 50 ft.c. and < 1 ft.c. when parallel to this plane.

According to the theory based on Fresnel's laws, the minimum amount of light will be refracted into the winkle's eyes when it is moving parallel to the plane of vibration and the maximum amount of light when its movements are at right angles to this plane. Thus with the coupled pointer apparatus it should be possible either to amplify or attenuate the natural response of a photonegative nut winkle to crawl parallel with the plane of vibration of the incident light.

All the trials were conducted in a dark room, and precautions taken to eliminate stray reflexions entering the experimental arena. In every trial the winkle was placed at the centre of the crawling surface facing in any direction. As soon as the winkle commenced to crawl the pointer was set parallel to its longitudinal axis and the operator provided the servo-mechanism by which this was maintained. The experiment was terminated when the animal reached the perimeter of the crawling surface. All mucus trails were carefully erased before the commencement of each trial. For the control treatments the light intensity was maintained constant at 50 ft.c. The same specimens were used both in the experimental treatments and in their relevant controls, and the trials were conducted alternately, with the experimental treatment preceding or following the control treatment at random, in order to minimize any effects induced by fatigue or habituation. Since the mucus tracks were recorded after the termination of each trial, there was no possible interference from the observer overlooking the crawling surface.

Results

The tracks were recorded and the results analysed as in the previous experiments with polarized light (Charles, 1961). The plane of vibration was maintained parallel with the reference axis throughout all the trials and the results expressed in the form of percentage distance crawled within 30° sectors either side of, and at right angles to, the reference axis.

Table 1 is a tabulation of the results obtained for one of the 'control' series of trials with polarized light of constant intensity, and column 2 shows the percentage of the total distances crawled within a 30° sector either side of the reference axis and column 3 the corresponding distances traversed within a 30° sector either side of the direction at right angles to the reference axis. The results were analysed statistically by applying 'Students' t test to ascertain whether the mean difference between the pairs of the percentages of the two equal angular groups were significantly greater than zero [Davies (1949) t test difference method].

Since it has already been established that *L. littoralis* (L.) orientates to polarized ght by crawling parallel to the *e* vector (Burdon-Jones & Charles, 1958*a*, *b*; Charles, 961), we were interested only in whether the tracks parallel to the plane of vibration xceeded those at right angles to it. As a difference in a particular sense was expected, single-tailed probability curve was employed for 'Students' *t* test. This was done y halving the probability value *P* obtained from the ordinary tables for *t*. [This nethod applies to all trials in this and the following section of this paper, where the vinkles were allowed to crawl freely over a comparatively large surface, with the xception of trials conducted in non-polarized light. Since a difference in a particular ense was not anticipated under such conditions, a double-tailed probability curve was mployed for 'Students' *t* test.]

Table 1. Littorina littoralis (*L.*) *in air. Responses to polarized light, light intensity constant*

Winkle no.	% distance crawled 0–30°, 150–180°, 180–210°, 330–360° from reference axis (*e* vector)	% distance crawled 60–90°, 90–120°, 240–270°, 270–300° from reference axis	Difference
1	78·0	8·0	+70·0
2	75·0	41·7	+33·3
3	76·2	4·7	+71·5
4	90·0	5·0	+85·0
5	52·9	5·9	+47·0
6	42·8	30·6	+12·2
7	69·6	13·0	+56·6
8	52·4	4·8	+47·6
9	79·0	5·3	+73·7
10	79·1	12·5	+66·6
11	76·9	7·7	+69·2
12	78·3	4·3	+74·0
13	53·8	28·2	+25·6
14	69·6	8·7	+60·9
15	44·7	20·4	+24·3
		Mean difference	+54·5

Standard error (S.E.) associated with the mean difference, ±7·74 %.
Value of $t = 7·04$. Degrees of freedom $\phi = 14$.
Value of *P* is $< 0·001$, by the single-tail *t* test.

Table 2 summarizes the results obtained by varying the light intensity with respect o the orientation of the mollusc. The trials in section A served as a control for B, ne same individuals being used in the two treatments. Similarly, the trials in section C erved as a control for D, E and F.

In both sets of control experiments, A and C, the distance traversed by *L. littoralis* arallel to the reference axis was significantly greater than the distance crawled at ght angles to this plane ($P < 0·001$).

When the light was dimmed in proportion to the deviation of the animal from ne reference axis (conditions B and D, Table 2) the artificial shading should oppose ne Fresnel effect of polarized light, and so it will be seen that the distance traversed

parallel to the reference axis (column 2) fell below that obtained for the control and the distance crawled at right angles to the plane of vibration increased correspondingly (column 3). The significance of the difference was less in Expt. B, the value of P lying between 0·025 and 0·01. In Exp. D the orientation to polarized light was lost, crawling not being significantly different from random.

Table 2. Littorina littoralis (*L.*) *in air. Response to polarized light of constant and of varying light intensity*

Manipulation of light intensity	Average % distance crawled within a 30° sector each side of: The reference axis (*e* vector)	The plane at right angles to the reference axis	Mean difference	S.E. associated with the mean difference	Total no. of animals tested	t	ϕ	[illegible]
A. Held constant	67·89	13·39	+54·5	± 7·74	15	7·04	14	< 0·0
B. Dimmed as the animal orientates away from the reference axis	46·09	23·46	+22·63	± 8·98	15	2·52	14	< 0·0 > 0·0
C. Held constant	61·9	17·08	+44·82	± 6·15	10	7·28	9	< 0·0
D. Dimmed as the animal orientates away from the reference axis	37·3	33·83	+ 3·47	±11·33	10	0·306	9	< 0·4 > 0·3
E. Brightened as the animal orientates away from the reference axis	65·06	9·55	+55·51	± 7·95	10	6·98	9	< 0·0
F. Varied continuously at a constant rate irrespective of directional orientation	36·9	36·92	− 0·02	±12·21	10	0·0016	9	> 0·9

* Single-tail t test.

In the second set of trials two additional conditions were applied. In series E the light intensity was caused to be minimized when the winkles were orientating parallel to the reference axis and increased proportionately as they deviated from it, rising to maximum intensity when they were crawling at right angles to this plane. This, the opposite treatment from that for B and D, should augment the response to polarized light. It will be seen from Table 2 that the distance traversed parallel to the plane of vibration (column 2) increased slightly and the distance crawled at right angles to the plane of vibration decreased considerably, when compared with the corresponding figures for the control (section C). In series F the light intensity was varied continuously and at random irrespective of the direction of orientation of the mollusc. This completely destroyed their ability to orientate to the plane of vibration. The winkles moved equally in all directions, traversing almost identical distances within the two angular groups (columns 2 and 3).

Conclusions

These results show that controlled variations in the intensity of the incident polarized light with respect to the directional orientation of the animal had a considerable influence on their orientation. The Fresnel effect theory (Charles, 1961) postu-

lated that when *L. littoralis*, which is always photonegative, is orientated in a direction parallel to the plane of vibration of the *e* vector of polarized light, the minimum intensity of illumination will be refracted into the eyes and fall on the retina; conversely when it is orientated at right angles to this plane the maximum amount of light will fall on the retina.

When the intensity of the incident polarized light was controlled in such a way as to counteract the changes in light intensity produced by the Fresnel refraction mechanism, the efficiency of orientation was impaired though not always destroyed. When these controlled variations were used to augment such a refraction mechanism of orientation the efficiency of orientation was improved.

No doubt a more marked effect could have been obtained if it had been possible to decrease the time lag between the movements of the hand-operated pointer controlling the light intensity control and the turning movements of the winkle. The head of the mollusc is constantly swinging from side to side and the manual servo-control could only be aligned with the winkle's longitudinal axis when it had commenced a definite turn to the right or to the left. The mollusc was therefore still able to receive an indication of the direction of the *e* vector from the Fresnel effect by turning the head, before the servo-mechanism came into play.

However, when the intensity of the incident polarized light was varied continuously irrespective of the orientation of the animal, this drawback was overcome. The random orientation so produced confirmed the importance of intraocular refracted changes in light intensity in the winkle's orientation. The results show that polarized light is not detected as such since changes in the intensity of the incident illumination can augment or interfere with the orientation of the molluscs. Moreover, these intensity changes can be used to simulate the response to polarized light and it therefore seems probable that these are the cause of orientation to the plane of vibration. Since the Fresnel effect predicts such changes in intensity with regard to the light refracted into the eye, it would appear likely that this is the mechanism of orientation.

The influence of reflexions from the substratum on the orientation to plane polarized light in freely moving animals

In the initial experiments to determine the relative importance of substrate reflexions in the orientation of Littorinae to polarized light the apparatus was modified from the basic apparatus previously used (Charles, 1961). The modifications (Fig. 1) were intended to reduce reflexions from the crawling substrate and consequently limit any stimulus that the mollusc might receive from the non-uniform pattern of intensities produced when plane polarized light is reflected from a suitable surface (Kalmus, 1958, 1959; Baylor, 1959). Vertical parallel rays of polarized light give rise to reflexions with minimum intensities in a direction parallel with the plane of vibration and maximum intensities in a direction at right angles to this plane. It was conceivable that the photonegative *L. littoralis* by orientating parallel to the plane of vibration was in fact responding to this reflected pattern when crawling over the wet Perspex plate surface.

Apparatus and procedure

The original Perspex plate was replaced by a smooth sheet of glass *c.* $\frac{1}{8}$ in. in thickness, supported on the cylinder (*G*) 40 cm. in diameter (Fig. 1). The inner walls and floor (*H*) of the cylinder were blackened to reduce reflexions. The actual crawling area was screened from extraneous reflexions by the circular screen (*F*) and the wide annular mask (*I*). Comparative trials were also carried out using the original Perspex substratum.

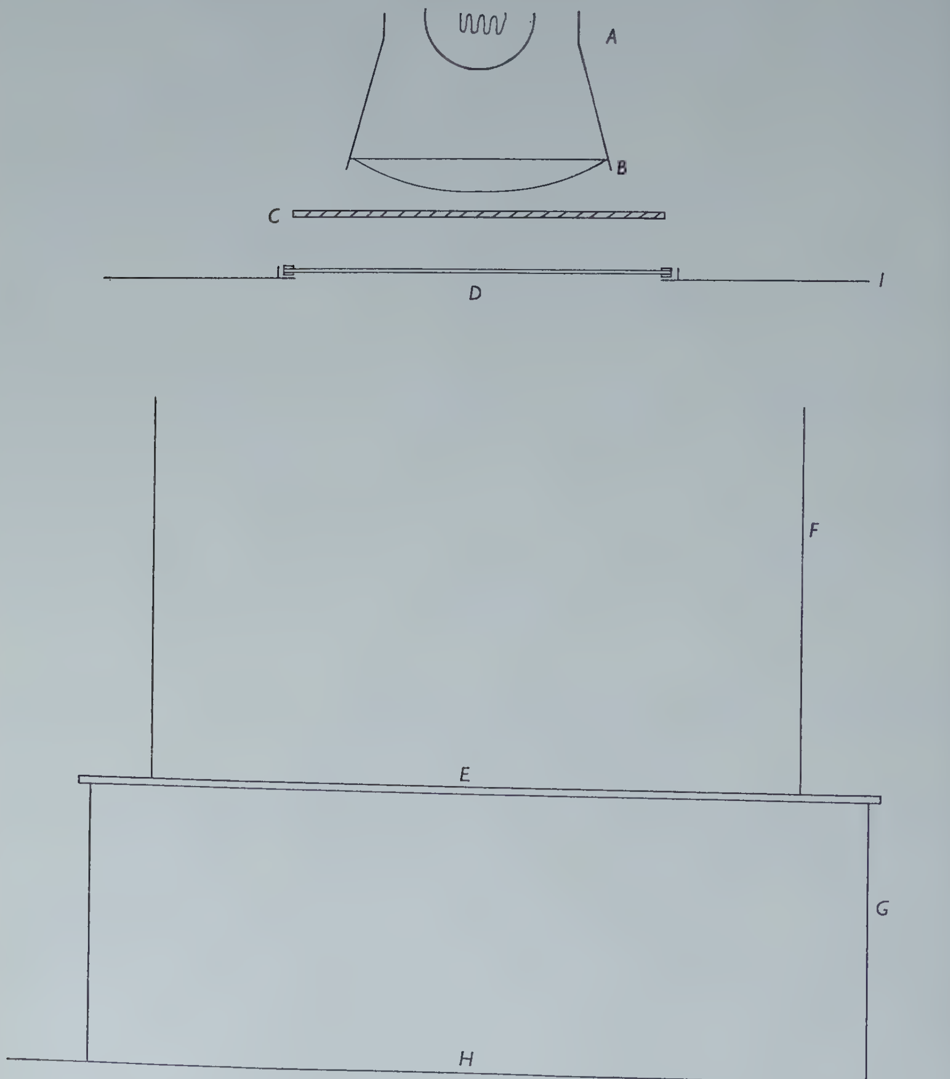

Fig. 1. Diagram of the apparatus used to test the responses of freely moving *Littorina littoralis* (L.) to plane polarized light, employing a clear glass sheet crawling surface suspended over a darkened cavity. *A*, 500 W. tungsten filament spotlamp; *B*, condenser; *C*, ground glass screen depolarizer; *D*, polaroid linear polarizer; *E*, clear glass sheet crawling-substratum; *F*, *G*, matt black cylindrical screens; *H*, matt black floor of the cylinder *G*; *I*, matt black annular mask.

Trials were also conducted on both types of surface using winkles which had their eyes shielded from all incident light from above. In so far as their morphology would allow they still possessed an unrestricted view of the substrate from below. The shields used (Fig. 2) permanently shaded the head, tentacles and eyes irrespective of the orientation of the animal. They were of negligible weight and permitted complete freedom of movement. All the experiments were conducted in a dark room. The sequence of each set of trials was varied alternately, the controls sometimes preceding and sometimes following the experimental treatments in order to eliminate any possible effects from fatigue or habituation.

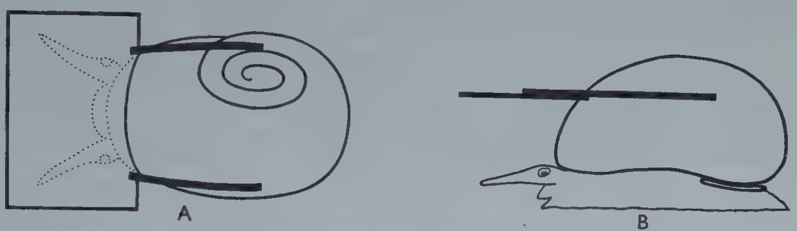

Fig. 2. Diagrams illustrating the matt black eye shield, 1·5 cm. × 1 cm., attached to the shell of *Littorina littoralis* (L.) by small clips. A. Dorsal view of winkle and eye shield. B. Lateral view of winkle and eye shield.

The procedure for each trial and the method of recording data was identical with that previously employed, but it was necessary to insert a grid beneath the glass substratum after each trial in order to plot the mucus trails that were left. *L. littoralis* was able to crawl over a dry glass surface without any difficulty.

Results

Treatments A–D of Table 3 summarizes the results obtained from the same specimens under different experimental conditions using the Perspex plate substrate.

Trials recorded in sections A and B were controls, using polarized and non-polarized light respectively. In both cases the vision of the winkles was unrestricted. The results were similar to those obtained in previous experiments under such conditions, *L. littoralis* orientating by crawling parallel to the plane of vibration of polarized light, but crawling at random in unpolarized light.

Although the orientation to polarized light was still quite significant ($P < 0{\cdot}01$, $> 0{\cdot}005$), shading the eyes from the incident light (treatment C) produced a definite decrease in the distance crawled parallel to the reference axis (column 4) and a corresponding increase in the distance traversed at right angles to it (column 5). A comparison was made between the mean difference crawled parallel to and at right angles to the reference axis, $+44{\cdot}98$ with the eyes unshaded and $+21{\cdot}01$ with the eyes shaded. Using the standard errors associated with these two measurements, the difference between them, namely 23·97, was compared with its standard error, and gave a value of P, for 56 degrees of freedom, of between 0·05 and 0·02 (Davies, 1949, t test, comparison of sample means).

The results obtained in non-polarized light (treatments D and B) showed that the presence of the eye shield, apart from shading the eyes, did not materially influence

Table 3. Littorina littoralis (*L.*) *in air. Responses to polarized light with eyes shielded from direct incident illumination from above, and with reduced reflexions from the crawling surface*

Treatments: Incident light	Treatments: Eyes	Crawling substrate	Average % distance crawled within a 30° sector from: The reference axis (*e* vector)	Average % distance crawled within a 30° sector from: The plane at right angles to the reference axis	Mean difference	S.E. associated with the mean difference	Total no. of animals tested	t	ϕ	P
A. Polarized	Unshielded	Perspex plate	58·55	13·57	+44·98	± 7·08	29	6·35	28	< 0·00
B. Non-polarized	Unshielded	Perspex plate	31·28	30·2	+ 1·08	± 6·02	28	0·18	27	< 0·9, > 0·8
C. Polarized	Shielded	Perspex plate	43·82	22·81	+21·01	± 8·15	29	2·58	28	< 0·01 > 0·00
D. Non-polarized	Shielded	Perspex plate	36·52	31·02	+ 5·5	± 6·87	26	0·8	25	< 0·5, > 0·4
E. Polarized	Unshielded	Perspex plate	55·8	14·98	+40·82	±10·28	26	3·97	25	< 0·00
F. Non-polarized	Unshielded	Perspex plate	32·88	32·45	+ 0·43	± 6·25	26	0·07	25	> 0·9
G. Polarized	Unshielded	Glass sheet	59·11	15·25	+43·86	± 6·25	28	7·02	27	< 0·00
H. Non-polarized	Unshielded	Glass sheet	29·17	44·36	−15·19	± 9·87	27	1·53	26	< 0·2, > 0·1
I. Polarized	Shielded	Glass sheet	49·48	20·39	+29·09	± 6·32	27	4·6	26	< 0·00
J. Non-polarized	Shielded	Glass sheet	31·48	35·11	− 3·63	± 6·64	26	0·55	25	< 0·6, > 0·5

* Single-tail t test.

the behaviour of the winkles when compared with the results obtained with the same illumination when the winkles were without eye shields. In both treatments the orientation was random, there being no significant difference between the figures in columns 4 and 5.

Treatments E–J were designed to compare the influence of a substratum which scattered a large proportion of the incident light (roughened Perspex) and one which reflected a smaller proportion of the light without scatter (glass sheet).

In treatments E and F the conditions using the rough Perspex surface were identical with those described for treatments A and B, Table 3, and the results were essentially the same.

When the same specimens were tested on the glass sheet (treatment *G*) a significant orientation to the plane of vibration was still obtained. The control treatment in non-polarized light (treatment *H*) showed no artifacts in the apparatus which would induce such an orientation, in fact there was a slight, though not significant, bias towards orientation at right angles to the plane of vibration.

The results of the trials when the winkles were fitted with eye shields and crawling on the glass substrate are recorded under treatments I and J. In polarized light (treatment I) *L. littoralis* still gave a significant response to the plane of vibration, the

proportion of distance crawled parallel to this plane (column 4) being well in excess of that traversed at right angles to it (column 5). However, the actual response was not as good as that recorded for the comparable trials with unshaded eyes (columns 4 and 5, treatment *G*). A comparison between the results for treatments G and I (the mean differences between the two angular sectors being +43·86 and +29·09 respectively) gave a value for t of 1·65 which for 53 degrees of freedom showed P to be between 0·2 and 0·1 (Davies, 1949, t test, comparison of sample means).

In non-polarized light and fitted with eye shields (treatment J) the winkles moved at random, no significant preference being shown between the two angular sectors (columns 4 and 5).

The differences between the crawling surfaces used, whether roughened Perspex or smooth glass, appeared to have no effect on the behaviour of the winkles. This can be seen by comparing column 6 for the pairs of treatments A and G, and C and I. In view of this the effect of the eye shields may legitimately be tested by combining the results on both types of surface. When this was done the mean difference in distance crawled parallel with and at right angles to the e vector for winkles with unshielded eyes (A and G) was 44·43 with a combined standard error of 4·69. When provided with eye shields the value of the mean difference (C and I) fell to 24·27 with a combined standard error of 5·14. The change in behaviour represented by the difference between these figures (43·73–24·74) was highly significant by the t test, with $P < 0{\cdot}001$.

Conclusions

The fact that the eye shields significantly reduced the response to polarized light shows clearly that the eyes are capable of the direct perception of the plane of vibration of polarized light probably as a result of the Fresnel effect at the eye surface.

The conclusion differs from that of Baylor (1959), who found that the marine snail *Nassa obsoleta* failed to respond to polarized light shining through a glass plate on to the mollusc which was crawling upside down beneath the glass plate. Baylor states that the overhead source of light was fully visible to *N. obsoleta*. I have not had the opportunity to examine this species, but in all the winkles studied here parallel light from directly below the animal would not shine on the lens aperture; even if it struck the eye at an oblique angle, the results might be very different from those expected with light shining from above.

It is also clear that after shading the eyes from the incident polarized light *L. littoralis*, like *N. obsoleta* (Baylor, 1959), is still capable of orientating to the pattern of light intensities reflected from the substratum, whether of roughened Perspex or of glass.

The reflexions from wet roughened Perspex would be expected to be greater, but since the rays are scattered in all directions the resulting pattern might not be as clear as that from a polished weakly reflecting glass surface, where the whole of the reflected light would show some pattern of light and shade. This would explain the more significant orientation obtained with treatment I (shielded eyes, glass sheet surface) in comparison with treatment C (shielded eyes, Perspex surface). No quantitative analysis was made of the brightness patterns reflected from the glass sheet

surface and no use was made of bloomed glass to reduce these reflexions, since an entirely new technique and form of apparatus was being evolved.

The eyes must clearly be capable of discriminating between comparatively slight gradations in light intensity. This conclusion is supported by earlier observations on the ability of winkles to distinguish, at considerable distances, intensity differences in the light reflected from a white background containing darkened areas, small in comparison to the total field of view. It is also further supported by the ability of *L. littoralis* to orientate accurately to polarized light of low intensities, below 1 ft.c. At this level differences between the intensities of the refracted light produced by the Fresnel effect must be comparatively small.

SUMMARY

1. The mechanism of orientation of *Littorina littoralis* (L.) to plane polarized light was investigated by means of a servo-mechanism, whereby the intensity of the polarized light was varied with the orientation of the winkle to the plane of vibration. It was shown that these controlled variations in light intensity augmented or decreased the efficiency of orientation. When the light was altered with change in orientation in a direction which would be expected to counteract the Fresnel reflexion/refraction mechanism a fall in responses to polarized light was observed. Random changes in the light intensity of the polarized beam also interfered with the orientation.

2. The influence of reflected patterns of light and shade from the surroundings on the orientation of *L. littoralis* to polarized light was investigated with the use of the eye shields and with surfaces of different reflective properties. It was conclusively shown that the winkles could orientate both by direct perception of the plane of vibration by the eyes and to comparatively small differences in light intensity in the pattern of light reflected from the substratum.

I would like to thank Dr C. Burdon-Jones for his help and advice in the supervision of this work, and Dr D. J. Crisp for many helpful suggestions and consideration of the statistical problems involved.

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ORIENTATIONAL MOVEMENTS OF THE FOOT OF *LITTORINA* SPECIES IN RELATION TO THE PLANE OF VIBRATION OF POLARIZED LIGHT

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INTRODUCTION

There is no doubt that extraocular analysis plays a part in the orientation of many animals to plane polarized light. Such an analysing mechanism is usually based on the light intensity gradients produced when polarized light is reflected from a suitable substratum and its importance has been stressed by several workers (Baylor & Smith, 1957, 1958; Baylor & Kennedy, 1958; Bainbridge & Waterman, 1958; and more recently by Baylor, 1959, and Kalmus, 1958, 1959).

Baylor (1959) showed that the orientation of *Nassa obsoleta* depends entirely on extraocular analysis, but Charles (1961 *a*) believed that *Littorina littoralis* (L.) (not *L. littorea* (L.) as misquoted by Baylor, 1959) was capable of detecting the plane of vibration of polarized light, both by means of substrate reflexions and direct perception through the eyes, because shading the eyes from incident polarized light diminished the response.

It was felt desirable to demonstrate a direct response to incident polarized light by removing surface reflexions altogether. An entirely new technique and form of apparatus was therefore devised.

Apparatus and procedure

The essential features of the new apparatus, shown in Fig. 1, were the use of fixed animals and the observation of orientational movements of the winkle's foot to determine its intended direction of crawling.

The winkle was held rigidly by a supporting rod (F) so that its foot rested on a small ball of paraffin wax (G) of such a size (*c.* 1 in. diameter for *Littorina littoralis*) that it could be rotated uniformly with the minimum of friction. The ball was floated on a column of sea water in the glass-bottomed cylinder (H), and the height of the water was adjusted so that no upward pressure was exerted on the animal's foot. Both the ball and surrounding cylinder were sufficiently small not to be included in the winkle's downward field of view. The surface of the ball was painted a matt white and uniformly stippled with small black dots. Any crawling movements executed by the foot of the animal were directly translated into a rotary movement of the ball. The dots made the direction of rotation of the ball readily apparent.

Forward crawling movements of the foot produced a rotation of the ball such that its upper surface moved in an antero-posterior direction in relation to the long axis

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of the animal. Likewise, when the winkle attempted to turn to the left or the right, the unequal pattern of muscle contractions in the foot, which normally brought about the change of direction, resulted in a clockwise or contra-clockwise rotation of the ball.

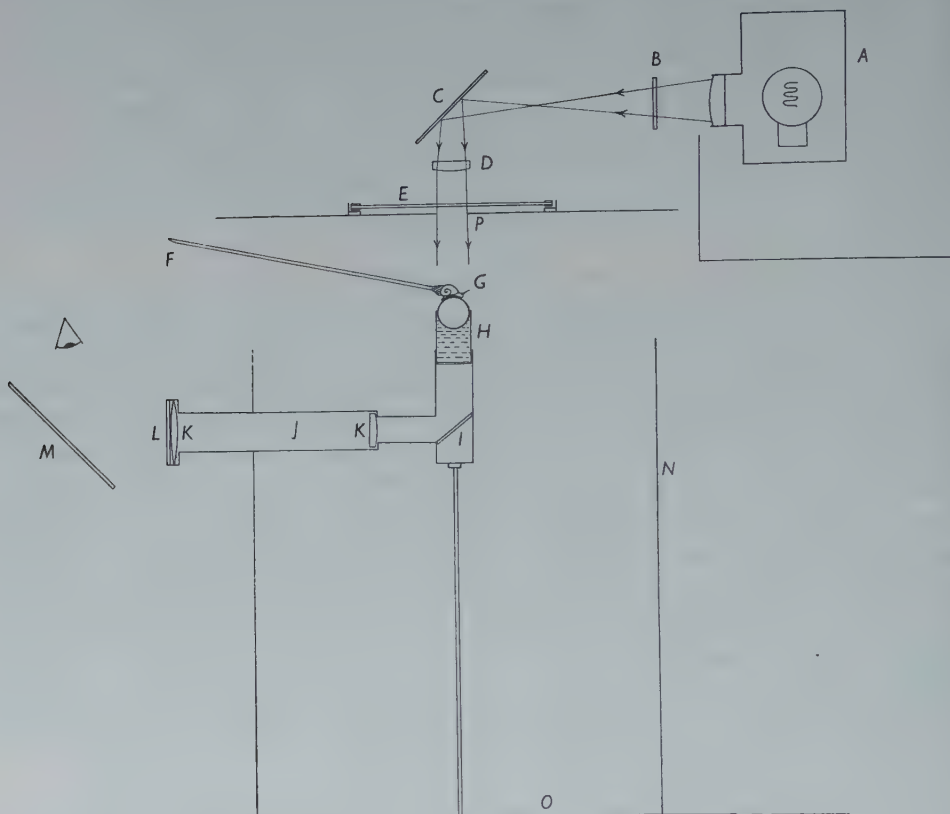

Fig. 1. Diagram of the apparatus used to reduce substrate reflexions of plane polarized light. *A*, 'Pointolite' light source with condenser, lamp housing screened from the rest of the apparatus; *B*, heat filter; *C*, plane mirror set at 45°; *D*, collimating lens; *E*, polaroid linear polarizer fitted in a rotatable housing; *F*, adjustable supporting rod; *G*, winkle rigidly attached by the rear of its shell to the rod (*F*) and with its foot in contact with a small wax ball; *H*, glass-bottomed cylinder, containing sea water and supporting the wax ball; *J*, arrangement of small tubes containing the plane mirror (*I*), lenses (*K*) and graticule (*L*); *M*, plane mirror in which an enlarged image of the lower hemisphere of the wax ball was viewed; *N*, matt black cylindrical screen; *O*, matt black floor surface; *P*, small aperture in the centre of a circular matt black screen, allowing the winkle to be illuminated with a narrow beam of polarized light.

Enough light passed between the ball and the rim of the cylinder (*H*) to illuminate the bottom hemisphere of the ball, an enlarged image of which was viewed against the eyepiece graticule (*L*) via a system of mirrors and lenses (*I*), (*K*) and (*M*). A 'Pointolite' was used as the source of illumination to reduce stray reflexions from the walls and floor of the dark room in which all trials were conducted. The light was adjusted so that the winkle was illuminated by a parallel beam of plane polarized light *c.* 3 cm. in diameter and 58 ft.c. in intensity, the minimum amount being allowed to pass beyond the cylinder (*H*). The amount of this stray light reflected from the blackened floor (*O*), tube (*J*) and cylindrical screen (*N*) was found by experiment to be

negligible in comparison with the amount of light directly incident upon the winkle (see below). As an extra precaution the rim and exterior of the cylinder (H) were painted matt white. Polarized light reflected from a matt white surface is largely depolarized, and most of the reflected light is scattered. In contrast a matt black surface, although reducing the amount of light reflected, would give this light a far more pronounced directional bias in relation to the plane of vibration of the incident rays (Kalmus, 1958).

The relation between the winkle's size and the diameter of the wax ball was fairly critical. Smaller specimens required a correspondingly smaller ball and supporting cylinder.

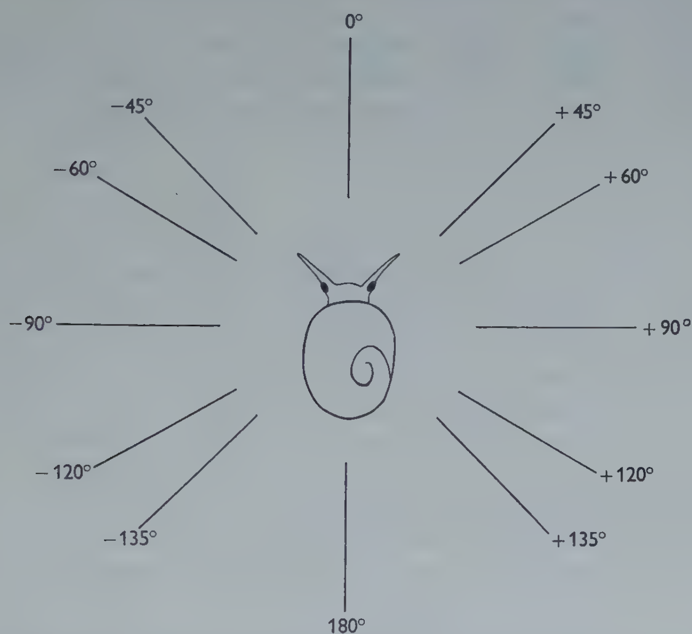

Fig. 2. Dorsal view of a winkle used in the trials with the apparatus shown in Fig. 1, illustrating the settings of the plane of vibration of the light employed, relative to the fixed longitudinal axis of the shell of the mollusc.

A line parallel to the fixed longitudinal axis of the winkle's shell and designated 0–180° was taken as the reference axis. The various settings of the plane of vibration of the light employed in the trials were referred to this axis (Fig. 2). The 0–180° setting was chosen to give the mollusc a symmetrical stimulation, and according to the Fresnel effect a photonegative winkle should endeavour to crawl parallel with the reference axis. Conversely, the −90°, +90° position of the plane of vibration should evoke a similar response in photopositive individuals. The 45°, 135° and 60°, 120° settings were selected to produce an asymmetrical stimulation and induce turning responses.

In a typical trial a winkle was attached to the adjustable supporting rod with the longitudinal axis of its shell parallel to the reference axis. The phototactic sign of each winkle was determined before and after each set of trials by illuminating it with a horizontal beam of ordinary light, first from one side and then the other at 90° to

the reference axis. This produced a turning response away from or towards the light source and a corresponding rotation of the ball. The sequence of trials with various treatments and controls were conducted in a random manner to overcome any possible effects induced by fatigue and habituation.

The results were recorded in the following manner. The winkle was illuminated with polarized light and the Polaroid set at one of the positions illustrated in Fig. 2. Each trial consisted of a fixed number of time periods, and the direction of rotation of the ball was recorded at the end of each period, which was indicated by an audible time signal. 40 periods of 10 sec. duration constituted one trial (except in the initial trials with *L. littoralis* when each trial comprised 20 periods of 20 sec. duration) and the results classified under three heads:

(1) 'Neutral' rotation of the ball, mollusc not attempting to turn. (N, Tables 1–4.)

(2) Clockwise rotation, indicating left-hand turning. (L, Tables 1–4.)

(3) Anticlockwise rotation, indicating right-hand turning of the winkle. (R, Tables 1–4.)

This enabled the relative proportions of the turning responses to be expressed numerically.

The neutral rotations were ignored and the number of left- and right-hand turns from each set of trials for one particular Polaroid setting were totalled, arranged as a contingency table and tested for significance by the χ^2 test. The expected value (null hypothesis) was in all cases assumed to be 50% in each direction of rotation, and allowance was made for continuity (continuity correction for the χ^2 test employing one degree of freedom, Davies, 1949).

It was necessary to apply two different forms of the χ^2 test according to the form of experiment.

When the plane of vibration was set parallel to or at right angles to the reference axis (Fig. 2) any movement of the head and tentacles to the left or to the right of the reference axis would produce the same small change in intensity of the light refracted into the eyes, since the effect of turning the head in either direction was symmetrical. Consequently, turning movements would be expected to occur with equal frequencies in either direction. Therefore the two tails of the probability curve should be used, the probability of the null hypothesis, viz. that there was no bias in either direction of turning, being given by the normal χ^2 tables, for one degree of freedom.

When the plane of vibration was set at any other angle, e.g. -45°, $+135^\circ$, to the reference axis (Fig. 2), a movement of the head and tentacles to the left would be expected to diminish the amount of light refracted into the eye, whereas a movement to the right (e.g. -40°, $+140^\circ$) would be expected to increase it. Hence the anticipated effect would no longer be symmetrical. It is therefore possible to predict the direction of turning left or right, which should predominate in a photonegative animal for -45°, $+135^\circ$ or any of the correct directions. In this case, therefore, we are interested only in whether the frequency of turning in the predicted direction can be accounted for by chance. If the frequency of turning predominated in the opposite sense to that expected the theory would be wrong. We therefore should use the single-tailed test with half the probability value given by the standard tables for χ^2.

RESULTS

Table 1 summarizes the results obtained for *L. littoralis* (L.), *L. saxatilis* (L.) and *L. neritoides* (L.). All the specimens used were photonegative.

Table 1. *Responses of* Littorina *species to plane polarized light with substrate reflexions reduced to a minimum*

Polaroid settings, plane of vibration at	Turning response N	Turning response L	Turning response R	Total no. of animals tested	χ^2 corrected for continuity	*P*
		***Littorina littoralis* (L.)**				
A. 0–180°	130	48	62	12	1·53	< 0·3, > 0·2
	54 %	20 %	26 %			
B. −45°, +135°	78	122	40	12	40·5	< 0·001*
	32 %	51 %	17 %			
C. +45°, −135°	71	35	134	12	56·83	< 0·001*
	29 %	15 %	56 %			
D. −60°, +120°	82	115	43	12	31·90	< 0·001*
	34 %	48 %	18 %			
E. +60°, −120°	69	40	131	12	47·37	< 0·001*
	29 %	17 %	54 %			
F. −90°, +90°	69	81	90	12	0·37	< 0·7, > 0·5
	29 %	34 %	37 %			
		***Littorina saxatilis* (L.)**				
A. 0–180°	244	78	78	10	0·0	1·00
	61 %	19·5 %	19·5 %			
B. −45°, +135°	168	178	54	10	65·21	< 0·001*
	42 %	44 %	14 %			
C. +45°, −135°	149	75	176	10	39·84	< 0·001*
	37 %	19 %	44 %			
D. −60°, +120°	139	186	75	10	46·36	< 0·001*
	35 %	46 %	19 %			
E. +60°, −120°	148	86	166	10	24·76	< 0·001*
	37 %	21 %	42 %			
F. −90°, +90°	151	124	125	10	0·0	1·00
	38 %	31 %	31 %			
		***Littorina neritoides* (L.)**				
A. 0–180°	608	141	131	22	0·29	< 0·7, > 0·5
	69 %	16 %	15 %			
B. −45°, +135°	458	301	121	22	75·92	< 0·001*
	52 %	34 %	14 %			
C. +45°, −135°	483	111	286	22	76·26	< 0·001*
	55 %	13 %	32 %			
D. −60°, +120°	413	278	109	22	72·92	< 0·001*
	47 %	32 %	21 %			
E. +60°, −120°	401	106	293	22	86·7	< 0·001*
	45 %	12 %	43 %			
F. −90°, +90°	424	240	216	22	1·16	< 0·3, > 0·2
	48 %	27 %	25 %			

N = 'Neutral' rotation of the ball; L = left-hand turning; R = right-hand turning.
* Single-tailed χ^2 test.

With the plane of vibration at 0–180° (treatment A) the frequency of 'neutral' responses was in excess of the frequency of the sum of the responses to the left (columns L) and to the right (columns R). These latter were of approximately equal frequency and the χ^2 test showed no significant difference. This result is interpreted as an attempt to crawl along a line parallel with the plane of vibration.

When the plane of vibration was at $-45°$, $+135°$ (treatment B) the proportion of neutral rotation fell and the amount of turning to the left (columns L) was significantly greater than to the right (columns R). The χ^2 test gave a highly significant value of $P < 0.0001$ for all species. The winkles were therefore trying to align themselves parallel with the plane of vibration of the light (cf. Fig. 2).

The results for treatment C with the plane of vibration set at $+45°$, $-135°$ were exactly similar with a significant turning response to the right.

Similar results were also obtained with the Polaroid settings $-60°$, $+120°$ (treatment D) and $+60°$, $-120°$ (treatment E).

When the plane of vibration was set at $-90°$, $+90°$, i.e. with the plane of vibration at right angles to the winkle's longitudinal axis and with symmetrical stimulation, there was no significant difference between the left- and right-hand turning responses (columns L and R). The winkles performed alternate twisting movements throughout 90° in an effort to align themselves parallel to the plane of vibration, and it can be seen that the frequency of neutral responses was much lower than in treatment A.

Experiments with Littorina littorea (*L*)

As previously described (Newell, 1958; Charles, 1961*b*), *L. littorea* exists in either a photopositive or photonegative phase. Table 2 summarizes the results obtained using fixed animals of this species which had previously been tested for their sign of response to light.

Photonegative specimens behaved in an identical fashion to *L. littoralis* by trying to orientate parallel to the plane of vibration (treatments A–D, Table 2).

The photopositive *L. littorea* (treatments A–D, Table 2) showed the same results as those previously obtained for freely moving photopositive periwinkles (Charles, 1961*b*) in trying to orientate at right angles to the plane of vibration.

The results for each of the treatments in Table 2 using photopositive *L. littorea* were the converse of those obtained with the photonegative periwinkles, using the same settings of the plane of vibration.

Comparative trials with Littorina littoralis *with shaded and unshaded eyes*

The eye shields used to shade the eyes of the winkles from the incident polarized light were identical with those previously described and used with freely crawling *L. littoralis* (Charles, 1961*a*). These shields, although shading the winkle's eyes, still permitted enough light to fall on those parts of the apparatus directly beneath the winkle. This enabled an experimental assessment of the efficiency of the apparatus in restricting stray reflexions to regions outside the winkle's downward field of view.

Table 3 summarizes the results obtained. The control treatments in which the eyes of the winkles were unshaded produced results identical with those obtained in the previous trials with *L. littoralis* and already given in Table 1.

Table 2. *Responses of* Littorina littorea (*L.*) *to plane polarized light, with substrate reflexions reduced to a minimum*

Polaroid settings, plane of vibration at	Turning response N	L	R	Total no. of animals tested	χ^2 corrected for continuity	*P*
	Littorina littorea (L.) photonegative					
A. 0–180°	245 61 %	78 19·5 %	77 19·5 %	10	0·0	1·0
B. −45°, +135°	178 44 %	169 43 %	53 13 %	10	59·57	< 0·001*
C. +45°, −135°	186 47 %	70 17 %	144 36 %	10	24·9	< 0·001*
D. −90°, +90°	176 44 %	115 29 %	109 27 %	10	0·11	< 0·8, > 0·7
	Littorina littorea (L.) photopositive					
A. 0–180°	165 41 %	113 28 %	122 31 %	10	0·27	< 0·7, > 0·5
B. −45°, +135°	159 40 %	76 19 %	165 41 %	10	32·13	< 0·001*
C. +45°, −135°	149 37 %	171 43 %	80 20 %	10	32·27	< 0·001*
D. −90°, +90°	209 52 %	102 25 %	89 23 %	10	0·75	< 0·5, > 0·3

N = 'Neutral' rotation of the ball; L = left-hand turning; R = right-hand turning.
* Single-tailed χ^2 test.

The photonegative specimens invariably attempted to align themselves parallel with the plane of vibration of the light when the latter was set at various angles to the reference axis (treatments B–E). In each case the asymmetrical stimulation produced a significantly greater tendency to turn to one side (right or left) more than the other. The values of *P* were all < 0·001, and when summed together, allowing for the signs of rotation of the Polaroid, the results were overwhelmingly significant (Table 3, Summary Table).

When the same specimens were tested with their eyes shaded from all incident polarized light from above, there was a general increase in the amount of 'neutral' movement for each of the treatments B–E. Furthermore, there was a complete absence of response to the plane of vibration. Even when all the results were combined, allowing for the signs of rotation of the Polaroid, the results were definitely not significant (Table 3, Summary Table).

Thus with this form of apparatus, shading the eyes completely destroyed the ability to orientate to the plane of vibration. Since it had been previously demonstrated that *L. littoralis* with shaded eyes could orientate to polarized light by means of the intensity gradients in substrate reflexions (Charles, 1961 *a*), it is contended that this apparatus effectively reduced such reflexions to a minimum.

Therefore *L. littoralis* is capable of orientating to polarized light directly incident upon the eyes.

Table 3. *Responses of* Littorina littoralis (*L.*) *to polarized light, with substrate reflexions reduced to a minimum*

Polaroid settings, plane of vibration at	Turning response N	L	R	Total no. of animals tested	χ^2 corrected for continuity	*P*
			Eyes unshaded			
A. 0–180°	210 52 %	90 23 %	100 25 %	10	0·43	< 0·7, > 0·5
B. −45°, +135°	140 35 %	190 47 %	70 18 %	10	54·46	< 0·001*
C. +45°, −135°	127 32 %	98 24 %	175 44 %	10	21·16	< 0·001*
D. −60°, +120°	160 40 %	177 44 %	63 16 %	10	53·2	< 0·001*
E. +60°, −120°	173 43 %	60 15 %	167 42 %	10	49·49	< 0·001*
			Eyes shaded			
A. 0–180°	185 46 %	102 25 %	113 29 %	10	0·46	< 0·5, > 0·3
B. −45°, +135°	200 50 %	113 28 %	87 22 %	10	3·12	< 0·05*, > 0·025
C. +45°, −135°	195 49 %	105 26 %	100 25 %	10	0·076	< 0·4*, > 0·35
D. −60°, +120°	185 46 %	112 28 %	103 26 %	10	0·29	< 0·35*, > 0·25
E. +60°, −120°	185 46 %	117 29 %	98 25 %	10	1·51	< 0·15*, > 0·1

N = 'Neutral' rotation of the ball; L = left-hand turning; R = right-hand turning.
* Single-tailed χ^2 test.

Summary Table. *Summary of all the results with treatments B, C, D and E*

	Turning in expected direction	Turning against expected direction	χ^2 corrected fo continuity	*P*
Eyes unshaded	709	291	173·88	< 0·001
Eyes shaded	423	412	0·1196	< 0·8, > 0·7

The influence of the angle of incidence on the orientation of Littorina littoralis (*L.*) *to plane polarized light*

Fresnel's laws of refraction of polarized light state that at all angles of incidence, save 0° and 90°, light vibrating in the plane defined by the incident and reflected rays will be more efficiently refracted than light vibrating in the plane at right angles to this. The difference in intensity of the refracted light caused by the angle subtended by the plane of vibration decreases progressively and finally disappears when the angle of incidence of the polarized rays becomes zero (Stephens, Fingerman & Brown, 1953; Waterman, 1954).

The theory previously put forward to account for the orientation of the Littorinae to polarized light, based on Fresnel's laws of refraction (Burdon-Jones & Charles, 1958;

Charles, 1961*b*), predicts therefore that if the angle of incidence of the polarized light on the surface of the eyes were 0° no orientation should be possible.

The apparatus and technique employing fixed animals and a rotating wax ball is particularly suitable for testing this prediction.

The apparatus illustrated in Fig. 1 was modified by the insertion of two pivots in the supporting framework of the lamp, mirror and Polaroid housing. These pivots were set in such a way that the light beam could be inclined at an angle to the sagittal plane of the winkle, by being moved through an arc the plane of which was at right angles to the saggital plane.

For the experimental treatments the beam was tilted to subtend an angle of 45° to the vertical axis of the mollusc. The angle of incidence of the greater part of the eye surface of the mollusc would then be of the order of only 5°. The beam would still be centred on the mollusc whether directed vertically or inclined.

Since tilting the beam would inevitably cause unequal illumination of the eyes, unilaterally blinded *L. littoralis* were employed in these trials. The entire left or right eye was excised from winkles which had been previously narcotized with a 7·5% solution of magnesium chloride in sea water. After a period of 2–3 days the wounds healed and all molluscs made a complete recovery. These animals were not entirely normal in behaviour and tended to circle towards the blind side; they could, however, still discriminate between light and shade and could locate small dark areas in an illuminated arena.

Winkles with their left eyes excised were tested with the beam vertical and tilted so that their right eyes were illuminated; and those blinded on their right-hand sides, *mutatis mutandis*.

Otherwise the procedure for the trials and the method of recording and analysing the results were the same as described in the previous section.

The results are summarized in Table 4. The results from the trials with winkles blinded on their left and on their right side were exactly symmetrical. Therefore only those results for animals blinded on the left side will be considered in detail.

When the plane of vibration was set at 0–180° (cf. Fig. 2) and the light beam vertical (treatment A, α, Table 4) the winkles behaved as normal animals and endeavoured to crawl parallel to the plane of vibration. There was no significant difference between the frequency of left- and right-hand turning and there were no circus movements. Freely moving unilaterally blinded animals were also free of circus movements in the plane polarized light.

With the beam tilted, however, but with the plane of vibration still parallel to the long axis of the mollusc, the frequency of turning to the right tended to be significantly greater than that to the left (treatment A, β, Table 4). This result was surprising, and the reason for it is not at present understood.

With the plane of vibration set at −45°, +135° and the light beam vertical (treatment B, α) a significant turning response to the left was obtained in the expected direction ($P < 0{\cdot}001$). However, on decreasing the angle of incidence by tilting the beam (treatment B, β) the efficiency of orientation decreased and there was little difference between the two possible turning responses (columns L and R) ($P < 0{\cdot}5$, $> 0{\cdot}1$).

Table 4. Littorina littoralis (*L.*). *Responses to polarized light with decreased angle of incidence of the light upon the eyes*

Polaroid settings, plane of vibration at	Turning response N	L	R	Total no. of animals tested	χ^2 corrected for continuity	*P*
		α. Left eye excised, light beam vertical				
A. 0–180°	232 58 %	82 20 %	86 22 %	10	0·053	< 0·9, > 0·8
B. −45°, +135°	154 38 %	166 42 %	80 20 %	10	29·36	< 0·001*
C. +45°, −135°	171 43 %	69 17 %	160 40 %	10	35·36	< 0·001*
		β. Left eye excised, light beam at 45° to the vertical				
A. 0–180°	188 47 %	86 22 %	126 31 %	10	7·17	< 0·01, > 0·001
B. −45°, +135°	166 41 %	127 32 %	107 27 %	10	1·54	< 0·15*, > 0·1
C. +45°, −135°	167 42 %	121 30 %	112 28 %	10	0·27	< 0·35*, < 0·25
		γ. Right eye excised, light beam vertical				
A. 0–180°	224 56 %	96 24 %	80 20 %	10	1·28	< 0·3, > 0·2
B. −45°, +135°	181 45 %	172 43 %	47 12 %	10	51·94	< 0·001*
C. +45°, −135°	178 45 %	48 12 %	174 43 %	10	70·38	< 0·001*
		δ. Right eye excised, light beam at 45° to the vertical				
A. 0–180°	163 41 %	160 40 %	77 19 %	10	27·0	< 0·001
B. −45°, +135°	188 47 %	101 25 %	111 28 %	10	0·38	< 0·35*, > 0·25
C. +45°, −135°	211 53 %	90 22 %	99 25 %	10	0·34	< 0·35*, > 0·25

N = 'Neutral' rotation of the ball; L = left-hand turning; R = right-hand turning.
* Single tailed χ^2 test.

Conversely, when the Polaroid was set at +45°, −135° (treatment C, *α*) a significant turning response was again obtained in the expected direction with the beam in the vertical position, but the effect disappeared when the beam was tilted to 45° and the angle of incidence decreased (treatment C, *β*).

CONCLUSIONS

These trials show conclusively that *L. littoralis*, *L. saxatilis*, *L. neritoides* and *L. littorea* can, in the absence of the pattern of light intensities produced when plane polarized light is reflected from a suitable substrate, orientate directly to the plane of vibration of the polarized light incident upon the winkle from above. Photonegative winkles will orientate themselves parallel with, and photopositive winkles at right angles to, the plane of vibration (*e* vector) of the light.

When the angle of incidence of polarized light falling on to the surface of the eyes of *L. littoralis* is reduced towards zero, the ability of the winkle to orientate to the plane of vibration disappears. It is a reasonable supposition that this is brought about by diminishing the differences between the intensities of light refracted into the optic cup relative to the plane of vibration, and these results are put forward as further proof of the reflexion/refraction mechanism of orientation. These results afford an interesting comparison with those obtained by Waterman (1954) with the angle of stimulus incidence of plane polarized light on single ommatidia from the compound eye of *Limulus*. He found that when the rays of polarized light entered an ommatidium parallel to its optical axis and normal to the corneal surface, little or no polarized light sensitivity was obtained. This was in contrast to the ability of the ommatidia to detect the plane of vibration when the angle of incidence of the rays was large and struck the surface of the cornea at an oblique angle. Waterman (1954) suggests the possibility of a Fresnel reflexion/refraction mechanism in the polarized-light sensitivity of *Limulus*.

An interesting feature of the experiments with *L. littoralis* was that unilaterally blinded winkles were able to give an equally good response to the plane of vibration of a vertically directed beam as winkles with both eyes intact. This fact, together with the observation that unilaterally blinded winkles can orientate to a small dark area, shows that orientation does not depend on balanced stimulation of the two eyes. It seems likely that the recognition of the plane of vibration of polarized light and of light and dark areas of the visual field is achieved by the changes in light intensity produced on the retina as the animal swings the head and tentacles from side to side.

Since the Fresnel reflexion/refraction phenomena occur at the interphase between the eye surface and the external medium, it is debatable whether the term intraocular perception can be used in this context. However, the concentric laminae of the lens provide numerous interphases which contribute to the Fresnel effect (Charles, 1961 *b*) and in this sense the mechanism is partially intraocular. Some distinction should be made between this method of analysing polarized light and purely extraocular perception, such as responses to brightness patterns in substrate reflexions.

The role, if any, that polarized light orientation plays in the life of winkles on the shore has yet to be investigated. It is theoretically possible for the winkle to respond to polarized light from the sky, but responses to reflected substrate patterns may be of greater importance. Reflected patterns of polarized light have their maximum brightness in the same azimuth as the sun and these could play a part in the light compass/sun orientation responses exhibited by the Littorinae.

SUMMARY

1. *Littorina littoralis* (L.), *L. saxatilis* (L.), *L. neritoides* (L.) and *L. littorea* (L.) responded to the plane of vibration (*e* vector) of polarized light directly incident upon their eyes in the virtual absence of any substrate reflexions. Photonegative winkles orientated parallel with the plane of vibration and photopositive individuals at right angles to this plane.

2. Decrease of the angle of incidence towards zero of polarized light rays incident upon the lens aperture of *Littorina littoralis* completely destroyed the ability to

orientate to the plane of vibration. This can be explained on the basis of a Fresnel reflexion/refraction mechanism of orientation.

3. Unilaterally blinded *Littorina littoralis* gave just as good an orientation to vertical rays of polarized light as winkles with intact vision.

4. It is concluded that the eyes of *Littorina* species are capable of detecting the plane of vibration of polarized light directly incident from above by means of a Fresnel reflexion/refraction mechanism, and that orientation does not depend on balanced stimulation of the two eyes.

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THE RELATIONSHIP BETWEEN NUTRITION, HORMONES AND REPRODUCTION IN THE BLOWFLY *CALLIPHORA ERYTHROCEPHALA* (MEIG.)

I. SELECTIVE FEEDING IN RELATION TO THE REPRODUCTIVE CYCLE, THE CORPUS ALLATUM VOLUME AND FERTILIZATION

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INTRODUCTION AND METHODS

Wigglesworth (1936) showed that the corpus allatum was necessary for ovarian development in *Rhodnius prolixus* females, and this has been confirmed in many other insects (Wigglesworth, 1954). Thomsen (1940, 1942) confirmed these results in *Calliphora erythrocephala* and (1948, 1952) found that the median neurosecretory cells of the protocerebrum were also necessary for egg development. Fraenkel (1940) found that *Calliphora* females failed to develop their ovaries if protein-containing substances were omitted from the diet, but that the flies continued to live provided sugar and water were ingested. If the flies were fed protein-containing substances but no carbohydrate, they died. The fact that these three phenomena—nutrition, hormones and reproduction—appeared to be connected, led the author to investigate their possible relationships.

The blowfly *Calliphora erythrocephala* was chosen as the experimental insect for several reasons. It was easily cultured (maintained on a diet of meat, sugar and water at 25° C. with constant lighting from two 40 W. fluorescent tubes) and was already available in the laboratory. Its reproductive-endocrine relationships had already been investigated (see above) which eliminated the necessity of confirming fairly well established facts. Its reproductive cycle was conveniently rapid yet slow enough to allow recognition of the different phases within the reproductive cycle. Finally, females could be maintained in a reproducing or non-reproducing condition merely by controlling their diet.

Since the ability to reproduce depended upon nutrition, it was considered possible that females would select food in proportions which would vary with the state of their ovaries. A technique was therefore required whereby one could measure food selection and relate this to the state of the ovaries. The ideal requirements for measuring food intake were considered to be: (1) that females should be able to select from different foods at any time throughout each 24 h. day period; (2) that ingestion of these foods should be measurable at any and at all times; (3) that individual food selections should be traceable to individual females; (4) that the above three conditions should be synchronously applicable to a large number of flies. These requirements were fulfilled in the following manner.

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Females were isolated in 3×1 in. glass tubes (Fig. 1). One end of each tube was covered by nylon netting and the other was stoppered with a cork which had first been plugged into a hole in a Perspex tray. Two thick-walled capillary tubes projected through holes in the cork. The Perspex tray was held just off the vertical by a wooden frame (Fig. 2), and thus the capillary tubes (which projected at right angles to the tray) lay just off the horizontal. The tray could hold 100 feeding units, and the entire assembly fitted into an incubator maintained at 25° C. with constant lighting from two 40 W. fluorescent tubes. The latter were positioned between the internal glass and external wooden doors of the incubator. The sides of the incubator had been extended to provide the necessary space for the fluorescent tubes. The openings above and below these extensions allowed air to circulate and this prevented overheating of the incubator.

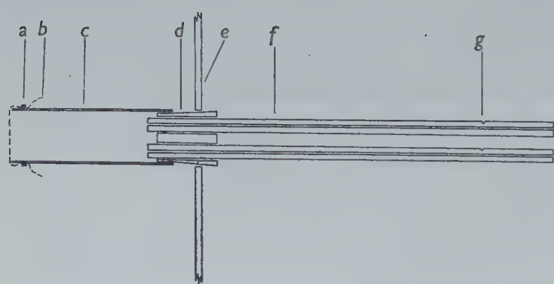

Fig. 1

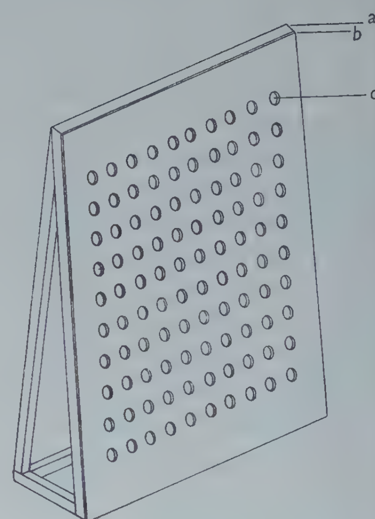

Fig. 2

Fig. 1. A selective feeding unit viewed in section from above. *a*, Rubber band; *b*, nylon netting; *c*, a 3×1 in. glass tube; *d*, a cork with two holes bored through it; *e*, Perspex tray; *f*, capillary tube; *g*, file mark up to which the capillary tube is filled with food solution.

Fig. 2. Feeding unit stand. *a*, Wooden frame; *b*, Perspex tray; *c*, hole for holding the cork of a feeding unit.

The capillary tubes were filled to a file mark with two solutions: (*a*) 5 g. Marmite (a yeast extract whose composition is tabulated in a paper by Harlow, 1956) in 20 g. milk, which was made up daily. This mixture was known to be adequate for the ovarian development in the blowfly *Protophormia terrae-novae* (Harlow, 1956) and a preliminary trial had shown it to be adequate in *Calliphora*. This protein-containing solution will be referred to as 'protein' hereafter. (*b*) 100 g. sugar in 100 ml. water. The solutions held in the capillary tubes were prevented from overflowing at the lower ends by surface tension and yet when solution was removed by ingestion, gravity caused the liquids to flow down. Flies were thus presented with a constant source of the two types of food required for maintenance and reproduction, and the amounts ingested could be measured from the file marks to which the capillary tubes had been

filled. Readings were taken in the mornings and late afternoons when the tubes were washed, dried and refilled. The tubes were washed and dried by attaching them to rubber tubes clamped to water and compressed-air taps respectively. The solutions were drawn up the tubes with the help of a rubber teat. The capillary tubes had an average internal diameter of 0·041 cm. Since measurements of only relative quantities were required, any slight differences between the tubes of different flies would not affect the results obtained because each fly was given the same tube containing the same food throughout the experiment.

To determine losses by evaporation three control units were used in all experiments, and these were identical in all respects to the normal units except that no flies were placed in the glass tubes. In all subsequent graphs, ingestion readings are for complete 24 hr. periods, allowance having been made for evaporation.

SELECTIVE FEEDING IN RELATION TO THE REPRODUCTIVE CYCLE AND THE VOLUME OF THE CORPUS ALLATUM

It was thought that variations in ingestion were probably related to phases within the reproductive cycle. As no means of assessing the stage of the reproductive cycle without harming the fly was then known, it was necessary to design the experiment so as to allow for the daily dissection of several flies. The stage of the ovaries could then be related to ingestion up to that moment.

Nine females were removed from a stock culture daily and were isolated in feeding units. Five of these were allowed to select from two capillary tubes as described above, two were given only one tube containing carbohydrate solution but were also given fresh meat, and two were given the 'protein' solution in a capillary tube but were also given carbohydrate in the form of sugar lumps. Thus any effect caused by ingesting a large volume of one solution upon the ingestion of the other was expected to become apparent by comparison with the control flies with either solid meat or sugar. Since the volumes of solutions ingested by the control flies were not noticeably greater than the volumes of the same foods ingested by the experimental flies (Figs. 3–6), it was assumed that the latter ingested (selectively) both solutions in quantities sufficient for (and determined by) their requirements.

After 24 hr. these flies were replaced by nine others and were then dissected for examination of the corpora allata (c.a.) and the ovaries. The c.a. were removed using the technique used by Thomsen (1942). Each c.a. was measured in arbitrary units along its length, breadth and depth and the product of these measurements was used for relative comparison. The longest and shortest egg in each fly was measured lengthwise and the mean was used for comparison.

In all, sixty-three females were investigated over an age range of 4–11 days. The results were as follows.

Protein ingestion. 'Protein' ingestion is plotted against egg length in Fig. 3, but the considerable scatter of points on the graph prevents any convincing interpretation. Similarly, there does not appear to be any correlation between c.a. volume and 'protein' ingestion (Fig. 4).

Carbohydrate ingestion. No correlation is apparent between carbohydrate ingestion and egg length (Fig. 5), nor between carbohydrate ingestion and c.a. volume (Fig. 6).

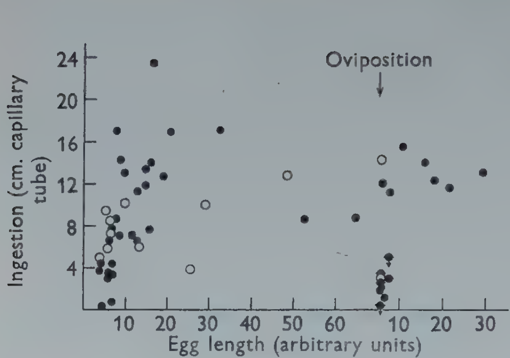

Fig. 3

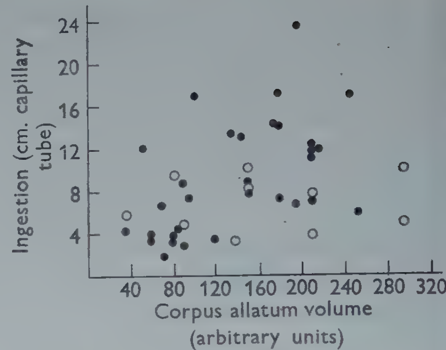

Fig. 4

Fig. 3. Protein ingestion in relation to egg length. Females were fed on: ●, solutions of protein and carbohydrate; ○, protein solution and solid sugar lumps. -●- and -○-, as above except that measurements are of the oocytes adjacent to mature eggs. These latter measurements are necessary because reproductive cycles tend to overlap. The natural sequence of events is therefore lost unless the succeeding cycle is taken into consideration. ↓, These females were ovipositing just before being placed in feeding units.

Fig. 4. Protein ingestion in relation to corpus allatum volume. Females were fed on: ●, solutions of protein and carbohydrate; ○, protein solution and sugar lumps.

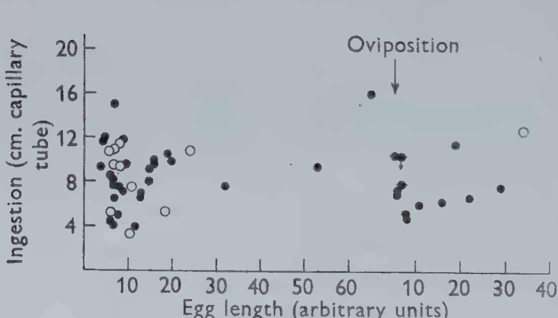

Fig. 5

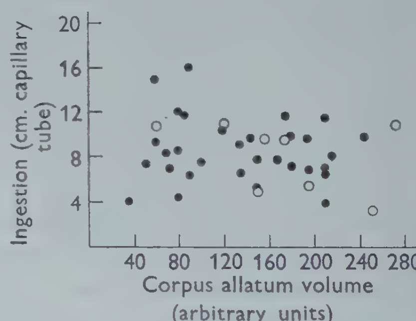

Fig. 6

Fig. 5. Carbohydrate ingestion in relation to egg length. Females were fed on: ○, carbohydrate solution and solid meat; -●-, and -○-, as above except that measurements are of the oocytes adjacent to mature eggs; ↓, these flies were ovipositing immediately before being placed in feeding units.

Fig. 6. Carbohydrate ingestion in relation to corpus allatum volume. Females were fed on: ●, solutions of carbohydrate and protein; ○, carbohydrate solution and solid meat.

The almost complete absence of correlation between any of the factors mentioned above is of considerable interest and importance. For, as is shown below, a relationship between these four factors does exist, and the reason why these relationships are not evident in the present graphs might explain why other authors have failed to find relationships between phenomena which one might expect to be related (cf. Thomsen & Hamburger (1955); these authors were unable to find a relationship between c.a. volume and oxygen consumption and yet were able to demonstrate that the c.a. did influence oxygen intake). The implications of these results will be discussed in a later paper in this series in conjunction with additional data.

It seemed unlikely that neither protein nor carbohydrate ingestion should be influenced by phases of the reproductive cycle. It was therefore considered possible that this might have been due to individual variations in ingestion. In an attempt to eliminate these variations, egg lengths were plotted against the ratio of carbohydrate to 'protein' (C/P ratio) ingested (Fig. 7). Selective feeding (C/P ratio) then showed a noticeable cyclical fluctuation which was correlated with the reproductive cycle. The graph shows the C/P ratio to be high for a short time after emergence, but as the oocytes grow and as the remains of the pupal protein stores are utilized (which takes 3–4 days), the ratio drops and remains low until the beginning of yolk deposition when the ratio begins to rise, at first gradually and then more steeply to reach a

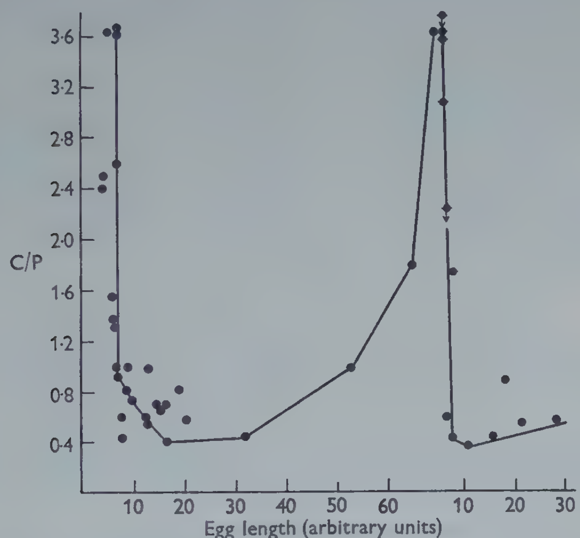

Fig. 7. The relation between selective feeding and egg length. ●, Normal measurements; -●-, measurements of oocytes adjacent to mature eggs; ↓, these females were ovipositing immediately before being placed in feeding units.

maximum by the completion of yolk deposition. Females which were removed from the stock culture whilst ovipositing are marked with a downward pointing arrow. The C/P ratios of these flies indicate that the C/P ratio drops just before, during or immediately after oviposition. The selective feeding cycle then begins again.

A comparison of the c.a. volumes with egg lengths confirmed the occurrence of a volume cycle (in preparation for publication) but here, as before, there was a considerable scatter.

In these initial experiments flies were removed from reproducing cultures and the problem of the influence of impregnation upon reproduction and selective feeding had not arisen. In the succeeding investigations, non-reproducing females (fed on sugar and water) were to be isolated before being given a reproducing diet and it was uncertain whether these would be fertilized. No literature was found describing the effects of impregnation upon reproduction in *Calliphora*, but the following few examples illustrate that mating often has a very definite effect upon reproduction.

Fertilization appears to be necessary for egg development in *Cimex* (Cragg, 1920, 1923; K. Mellanby, 1939), for normal ovulation in *Glossina* (H. Mellanby, 1937*a*, *b*) and in *Diploptera* (Engelmann, 1958), and for oviposition in *Lucilia* (Mackerras, 1933). Mating accelerates egg production in *Muscides* (Glaser, 1923), but in contrast, it appears to be unnecessary for egg production in *Haematopinus* (Florence, 1921) The effects of fertilization seem to vary and no general rule is apparent. It seemed essential to study these aspects before continuing with further investigations.

COURTSHIP IN RELATION TO NUTRITION AND REPRODUCTION

A reproducing culture was observed for considerable periods on several days. Males were often observed to attempt copulation with females, but in no instance was a female seen to approach or court a male. It appeared that males courted whilst females either accepted or rejected their advances.

Calliphora males are reported to be fully capable of fertilizing females a few hours after emergence (Lowne, 1892). Females, on the other hand, are unable to mature their ovaries for several days after emergence, and during this period protein must have been ingested. Since the time of fertilization appeared to depend upon the female rather than the male, experiments were designed to determine the conditions necessary for females to accept courting males.

The effect of a sugar diet upon courtship

Single pupae were isolated in corked glass tubes until emergence. This prevented possible copulation between newly emerged flies. On emergence three groups of five females were placed in separate 2 lb. jam jars (*a*), (*b*) and (*c*), with muslin covers. Into jar (*a*) were added five males which had emerged at the same time as the females. These might have been immature, so for comparison, five 'mature' males—taken from a week-old reproducing culture—were added to jar (*b*). As control, no males were added to jar (*c*). All jars contained sugar lumps and water. On the sixth day males were removed from the jars and meat was added to the diet. Females in each jar began ovipositing on the fourth day of the meat diet. Eggs were collected on this and the following 2 days and were kept for 7 days after oviposition. No larvae emerged from any of the three groups of eggs.

Assuming that some of the males were mature and that males normally initiate courtship, it would appear that *females which are fed on a sugar and water diet will not accept courting males.*

A meat diet was therefore considered to be necessary for copulation. Since protein consumption would result in ovarian development the second experiment was designed to determine the stage of ovarian development at which females would accept courting males.

Courtship in relation to ovarian development

As it had been concluded that copulation would not occur on a sugar diet, no attempt was made to separate the sexes at emergence. Males and females were allowed to emerge together in a cage and the flies were cultured for 6 days on a diet of sugar and water (culture 1). On the sixth day, 30 females were removed and were placed in a second cage (culture 2) to which was added 40 males. The latter were

obtained from a week-old reproducing culture and were used for comparison with culture 1. The comparison was thought to be necessary in order to determine whether an initial few days of sugar diet affected the abilities of males to fertilize females. Also on the sixth day, five females were removed from culture 1 and were placed in a 2 lb. muslin-covered jam jar containing sugar and water. These were to be control flies. Meat was then added to the diet of all flies.

Groups of five females were removed from each culture daily. Each group was placed in a separate jar and was fed sugar, meat and water. Eggs were collected daily and were retained for 7 days for observation. The results are tabulated in Table 1.

Table 1

Diet before isolation from males	Total diet until oviposition	Observations
	Culture 1	
6S	6S+4MS	No larvae
6S+1MS	6S+4MS	No larvae
6S+2MS	6S+5MS	No larvae
6S+3MS	6S+5MS	No larvae
6S+4MS	6S+5MS	Larvae hatched on day or day after oviposition
Stock culture 1	6S+4MS	
	Culture 2	
6S+1MS	6S+4MS	No larvae
6S+2MS	6S+4MS	No larvae
6S+3MS	6S+4MS	No larvae
6S+4MS	6S+5MS	Larvae hatched on day or day after oviposition
Stock culture 2	6S+4MS	

S = sugar; M = meat; 1–6 = days.

Results indicate that an initial sugar diet does not influence the fertility of males, and it is clear that *females are fertilized on the day of the first oviposition*, for only females which had been removed from the stock culture on the first day of oviposition (or later) produced eggs which hatched.

Since mating apparently did not influence the development of eggs, it was considered that mating would be unlikely to influence the selective feeding of unfertilized females and could therefore be ignored in future investigations.

FURTHER OBSERVATIONS ON SELECTIVE FEEDING IN RELATION TO REPRODUCTION

Selective feeding had been illustrated as a ratio (Fig. 7), but as such it gave no indication of the actual volume fluctuations. The C/P ratio curve could have been produced by simultaneous variations in the ingestion of both 'protein' and carbohydrate solutions or by variations in the consumption of either food whilst ingestion of the other remained constant. An experiment was therefore designed to clarify the picture.

Six females were removed from a sugar culture 10 days after emergence and were isolated in feeding units. Three of these were allowed to select from carbohydrate and 'protein' solutions and three were allowed only carbohydrate solution. The latter were intended as non-reproducing controls. The sugar control females survived the experiment, but one of the reproducing females died.

On the fifth day, as the C/P ratio began to rise, one of the remaining two 'protein'-ingesting flies was dissected for examination of the ovaries. As expected, ovarian development was in an early yolk-deposition stage. The third female was dissected on the eighth day, at the completion of the selective feeding cycle. It contained mature eggs. In Fig. 8, the flies which were fed on a mixed diet are shown individually and the carbohydrate controls are averaged. If a comparison is made between this graph and Fig. 7, it will be noticed that the C/P ratios in both graphs show a marked similarity in that they are low during the early stages of egg development and then rise steeply. Since the rise in Fig. 7 occurs during yolk formation, it is presumed that the rise in Fig. 8 corresponds to this stage, which occurs between the fourth and

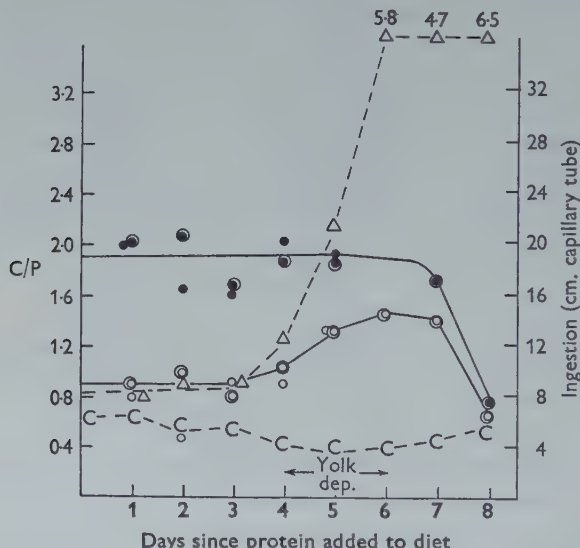

Fig. 8. Selective feeding in relation to days. • and ⦿, Total ingestion of individual females. ○ and ◎, Carbohydrate ingestion of the same two females respectively. The area between the total ingestion and carbohydrate ingestion represents protein ingestion. The female represented by • and ○ was dissected on the fifth day. △, Ratio of carbohydrate to protein (C/P) ingested by the female represented by ⦿ and ◎. C, Control non-reproducing females which were fed carbohydrate solution only.

sixth days. This supposition is supported by the presence of yolk-depositing eggs in the female dissected on the fifth day. It is therefore presumed that the first 4 days of selective feeding (in Fig. 8) represents a pre-yolk formation period, the fourth to the sixth days cover yolk deposition and the sixth to the eighth days include shell formation and delayed oviposition. In the succeeding experiments it was observed that females were unable to oviposit in the feeding tubes without meat, which would explain the constantly high C/P ratio in Fig. 8, for at oviposition (Fig. 7) the ratio drops and the cycle begins again.

Having related days to egg growth in Fig. 8 it is now possible to interpret selective feeding in terms of the reproductive cycle. The graph indicates that the reproductive cycle of females isolated in feeding units takes 7 days. The drop in total ingestion is considered to be a result of delayed oviposition (cf. Fig. 9). *The total volume of food ingested each day over a complete reproductive cycle is fairly constant. Within this total,*

however, 'protein' and carbohydrate are ingested in quantities which vary with different phases of the reproductive cycle. Thus, during the early stages of egg growth, 'protein' is ingested in relatively large quantities, while during yolk formation 'protein' ingestion declines. Carbohydrate ingestion, on the other hand, is relatively low during the early stages but increases during yolk formation, it remains high throughout what is possibly shell formation and then drops if oviposition is not possible. If oviposition were possible, then as carbohydrate ingestion dropped, 'protein' consumption would probably revert to the level typical of the pre-yolk phase (for the flies ingest meat whilst ovipositing), the C/P ratio would fall as shown in Fig. 7 and the selective feeding cycle would be repeated as is suggested in Fig. 9.

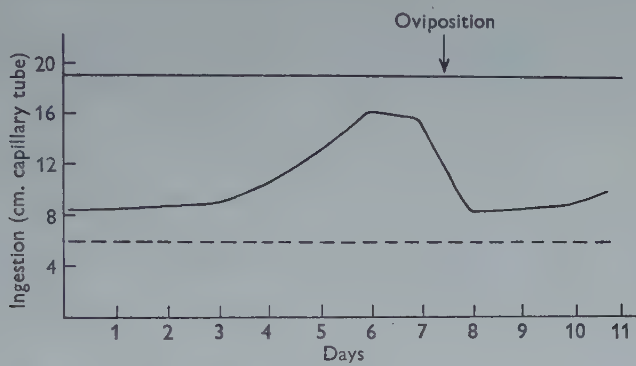

Fig. 9. Hypothetical recurrent selective feeding cycles. If it were possible to obtain recurrent selective feeding cycles, one might expect the above type of graph. Higher continuous line represents total ingestion. Lower continuous line represents carbohydrate ingestion. The area in between these two lines therefore represents protein consumption. Broken line represents control non-producing 'sugar' females. In the wild fly (Fig. 11) the 7-day to 8-day feeding cycle depicted above is condensed into 48 hr.

Attempts to obtain recurring selective feeding cycles failed because the flies would not oviposit on meat placed beyond their reach. When meat was within reach, the food was ingested as oviposition took place, so ingestion could not be measured. However, recurring carbohydrate cycles were obtained by providing a constant meat supply. The meat in these experiments was placed in an additional Perspex cylinder with a hole in its bottom which fitted tightly over the uncovered end of the fly compartment (Fig. 10). The other end of the meat container was covered by nylon netting which was held in position by the lid out of which a hole had been cut. It was noticed that the reproductive cycle was more rapid after the first oviposition. In wild flies (Fig. 11) eggs are deposited every second day and carbohydrate ingestion fluctuates sharply on alternate days.

In contrast to reproducing flies, non-reproducing females consume a constantly low volume of carbohydrate (Fig. 8) and there is no cycle.

Selective feeding was confirmed in ten other females, and further confirmation was obtained with the operated controls of succeeding experiments which will be described later.

An observation of considerable interest is that the initial large intake of 'protein'—at the beginning of the selective feeding cycle—is in addition to the normal volume of carbohydrate ingested (by comparison with the control females in Fig. 8). Thus,

carbohydrate intake is not depressed by protein intake and therefore the rise in the former during yolk formation is not a result from an earlier decrease and so must be a result of some other, possibly internal, inducement. The control of selective feeding will form the subject of a separate paper but has been briefly summarized already (Strangways-Dixon, 1959).

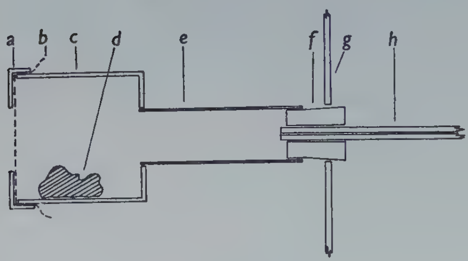

Fig. 10

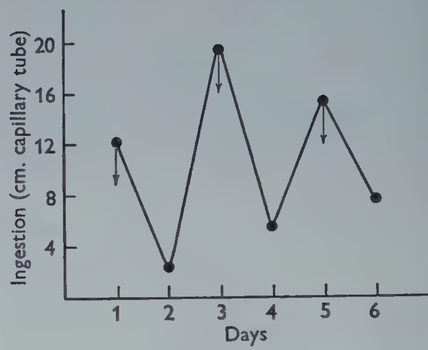

Fig. 11

Fig. 10. Feeding unit used when solid meat is made available. *a*, Lid with a hole cut in it; *b*, nylon netting; *c*, Perspex cylindrical meat chamber; *d*, meat; *e*, 3 in. × 1 in. glass tube; *f*, cork; *g*, Perspex stand; *h*, capillary tube.

Fig. 11. Recurrent carbohydrate ingestion cycles as shown by a wild fly which was also fed meat. ↓, Oviposition.

SUMMARY

1. *Calliphora erythrocephala* females will live on a sugar diet but will not reproduce unless protein-containing substances are also ingested. If fed protein without carbohydrate, they die.

2. Isolated females were allowed to select from a carbohydrate (sucrose) solution and from a protein-containing solution (Marmite yeast extract in milk) which were contained in identical capillary tubes. The total volume of food ingested each day over a complete reproductive cycle was found to be fairly constant. Within this total, however, 'protein' and carbohydrate were selected in quantities which varied with different phases of the reproductive cycle. Thus during the early stages of egg growth, 'protein' was ingested in relatively large quantities, while during yolk formation 'protein' ingestion declined. Carbohydrate consumption, on the other hand, was relatively low during the early stages but increased during yolk formation. At oviposition the cycles started again.

3. Carbohydrate ingestion of non-reproducing females (fed on sugar solution only) remained at a constantly low level.

4. Selective feeding did not appear to be influenced by mating.

5. Females were found to accept courting males on the day of the first oviposition but not before. This, of course, necessitated a mixed diet of carbohydrate and 'protein'.

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THE SPECTRAL SENSITIVITY OF *CALLIPHORA* MAGGOTS

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If fly larvae are put on a table by a window they immediately begin to travel away from the light. Turn the table round, and the maggots change direction so as to move directly away from the light again. They travel by a 'tacking' process—the head end is extended to right and left alternately, and each time the rear end is drawn after it. The animal moves on a straight course if this process is symmetrical; should a movement of the head bring it into a region of higher illumination or other undesirable condition the head is withdrawn, and a second step made to the opposite side, turning the animal round.

Placed between two opposing lights, the maggots will travel towards the weaker, and the direction of travel may be reversed by increasing the intensity of the weaker light until it, in turn, is the brighter. In the present work this reaction is used to equate the luminosity of lights of different wavelengths to a standard, and hence to draw a spectral sensitivity curve.

Bolwig (1946) performed a series of detailed experiments, behavioural and micro-anatomical, on the larva of the related *Musca domestica*, and says: 'The search for the light-sensitive cells has resulted in finding some cells at the bottom of a pair of pockets (directed forward) in the anterior end of the pharyngeal skeleton; these cells must undoubtedly be regarded as the light-sensitive cells.'

The gulf which separates the maggot from the adult fly, with its compound eyes and array of ocelli, is crossed in the 10 days or so of pupation. Ellsworth (1933) had thought that the tips of the maxillary lobes of the fleshfly larva were its light receptors, and that they must degenerate at metamorphosis without taking part in the formation of the compound eyes; this is not the case in larvae with lateral ocelli. Bolwig's finding, that these maxillary papillae are chemoreceptors in the housefly larva, means that the compound eye may evolve from the rudimentary lateral larval structure. It would be interesting to know if any light-sensitive pigment used by the maggot is retained through metamorphosis, or whether the emancipated fly has replaced the whole chemical basis of its larval light sense.

Much attention has been paid to the spectral sensitivity of adult blowflies, using behavioural methods (Schneider, 1956) and electrophysiological means (e.g. Walther & Dodt, 1959; Autrum, 1955). Apart from the work of Mast (1917) on unidentified blowfly maggots, and Bolwig (1946) on housefly larvae, however, there appears to be no information about spectral sensitivity of larval Diptera.

The wealth of information about adult flies appears rather inconsistent at first sight. Comparison of the various spectral sensitivity curves, which are illustrated in

Figs. 6 and 7, is reserved for the discussion; it will then be possible to include the present results, and to try to show an emerging pattern of dependence on intensity and other experimental conditions.

APPARATUS

A metal bridge was made, covered with black adhesive plastic film. The platform of the bridge was 12 in. long by 4 in. wide, and the sides were turned up to form 1½ in. high walls to prevent the maggots from straying. The ends of the platform were turned down to form sloping ramps 4 in. high, each leading down into a steep-walled dish to collect the maggots. Fig. 1 illustrates the experimental arrangement.

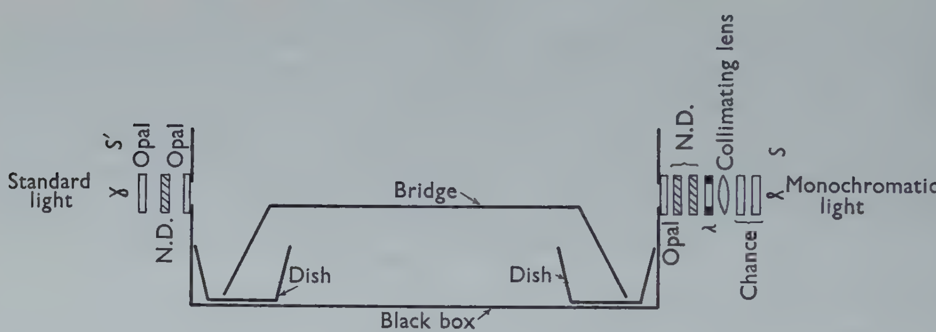

Fig. 1. A diagrammatic longitudinal section through the apparatus.

The bridge and the two dishes were placed symmetrically in a black box 17 in. long. In the end-walls of the box, on a level with the platform, were 2 cm. square holes filled with flashed-opal glass, illuminated respectively by the two light sources. Behind one opal square was a calibrated monochromatic light source. This consisted of a 32 V., 100 W. Philips projector lamp, *S*, fan-cooled. Two pieces of Chance glass absorbed heat from the lamp, an achromatic lens combination of focal length 1½ in. collimated the beam, which passed through a 2 cm. square Balzer interference filter, λ (type B, 40% transmission, the width of the band of wavelengths being about 10 mμ at the 20% transmission level), and through neutral density filters to the opal square. The wavelengths used in all the experiments were 402, 417, 442, 462, 489, 499, 513, 531, 554, 579 and 602 mμ. At the lower intensity level (level *B*) filters at 362, 392, 621 and 640 mμ were included. A filter at 470 mμ was added to the later series, at the higher intensity level (level *A*).

Suitable screening masks were provided, and the opal square was inspected from inside the black box before each run to confirm that stray light was excluded.

Behind the second opal square, at the other end of the bridge, was the standard light source, which consisted of a 12 V., 6 W. car headlamp bulb, *S'*, another piece of opal glass to diffuse the light, and a neutral density filter. (Both incandescent lamps were supplied by 50 cyc./sec. a.c., using suitable transformers.)

The standard light, without its neutral filter, gave a reading of 0·3 f.c. at the middle of the bridge, measured with a Holophane photometer. Two values of neutral filters were used, one (level *A*) giving approximately 0·01 f.c. at the middle of the bridge, and the other (level *B*) 0·001 f.c. (1 f.c. = 10·76 lumens per square metre).

The relative energy of the monochromatic source was found at each wavelength by a null method: the photo-current of a Mazda type 27M1 photomultiplier, of known relative spectral sensitivity, was kept constant by the addition of neutral density filters and neutral wedges.

The photomultiplier was calibrated at the National Physical Laboratory, and the nominally neutral filters and wedges were calibrated by comparison with a set of rotating sectors.

The same Ilford neutral density filters, in steps of approximately 0·25 log unit, were also used for the experiments.

MAGGOTS

The *Calliphora vomitoria* maggots, used in most of the experiments, were sold as 'gentles' for fishing. A sample of each batch was allowed to develop into flies, which were all identified as *C. vomitoria*.

The *C. erythrocephala* stock was kindly supplied by Prof. V. B. Wigglesworth.

The *C. vomitoria* were bred on stable manure, *C. erythrocephala* on lean beef. In both cases the animals were used after they had left their food, which was no longer visible in the alimentary tract.

METHOD

About forty maggots, dark-adapted for at least 2 hr., were placed in the middle of the bridge, using a wide funnel, in very faint light. A black cloth was spread over the outer box, and the two lights, one at each end of the bridge, were switched on simultaneously. After 10 min., the lights were switched off, and any maggots and pupae left on the bridge were discarded. The maggots in each dish were counted, and the number which had travelled towards the standard light was expressed as a percentage of the total in both dishes. (With the standard light alone, at least 70% travelled away from the light.) Each interference filter was used in combination with a series of neutral density filters, at approx. 0·25 log unit intervals; according to the intensity of this monochromatic light either more or less than half of the maggots went towards the standard light. An intensity was found by interpolation at which the two lights were equally effective, as in Fig. 2. The energy at each wavelength, matched like this to the same standard, is the relative threshold on an equal energy basis. To get the relative quantum threshold, the energy needed at each wavelength, λ, must be divided by the magnitude of a quantum of energy, which is proportional to $1/\lambda$.

The reciprocal of the relative quantum threshold, expressed as a percentage of its maximum, is the percentage quantum sensitivity. This function B, plotted against λ, may be considered a first approximation to the absorption spectrum of a hypothetical visual pigment.

RESULTS

In Fig. 3 the relative quantum sensitivity is plotted on a logarithmic base against wavelength. The points (●) refer to *C. vomitoria* at level A, and the circles (○) at level B. Crosses (×) refer to *C. erythrocephala*, tested only at level A.

The mean difference between the results at levels A and B for *C. vomitoria* was 1·04 log units, and this has been allowed for in plotting the curves.

The results at level B, (○) and (×), are derived from between four and ten groups of maggots, as in Fig. 2, which gives an accuracy of about $\pm 0{\cdot}1$ log unit. In order to

find the maximum more exactly for *C. vomitoria* at level *A* at least 15 groups of maggots were used to determine each of the points around 500 mμ, and 32 groups for the wavelength 499 mμ which was taken as 100% (2·0 on the logarithmic scale). The three sets of results are, within experimental accuracy, the same.

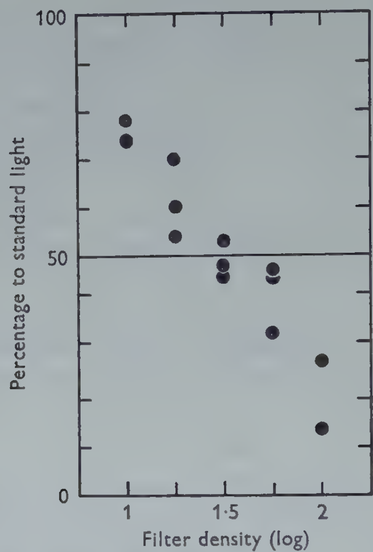

Fig. 2. Results of tests done on one day at 499 mμ. Each point represents the result for a group of maggots placed between the standard light and a given intensity of the monochromatic light, determined by the neutral density filter shown in the abscissa. When the monochromatic light is more effective, over 50% travel towards the standard light.

The lower level of illumination, level *B*, was used in order to extend the wavelength range (362–640 mμ). At level *A* it was restricted (from 402 to 602 mμ) by the energy of the monochromatic source, but this level had the advantage that the maggots could be used when their response to light was not at its strongest.

Fig. 4 shows the percentage quantum sensitivity for *C. vomitoria* plotted against wavelength. As before (●) represents results at level *A*, supplemented at lower and higher wavelengths by results at level *B*, shown as circles (○).

The bell-shaped curve is characteristic of visual pigments of the rhodopsin type, otherwise known as visual purple; assuming it to be due to a single pigment, it would be best fitted by a visual pigment with a maximum at 504 mμ. The absorption curve of such a hypothetical pigment is drawn (Dartnall, 1953).

The effect of the epithelium covering the light-sensitive cells is not known. The whole maggot is creamy white, so the most likely effect is to reduce the sensitivity to the violet end of the spectrum a little, by scattering. Bolwig does not describe any specialized structure to concentrate the light in *Musca domestica* maggots.

It was of interest to know the level of the relative threshold above the absolute threshold, and this was found for one wavelength. About 400 maggots of *C. vomitoria* were divided into eight groups on the last day before pupating, and were dark adapted.

The filter of wavelength 442 mμ was used, with 1, 2, 3, 4, 4·25, 4·5, 4·75 or 5 log units of neutral density filters. There was no opposing standard light, but otherwise the apparatus was the same as in the main experiments.

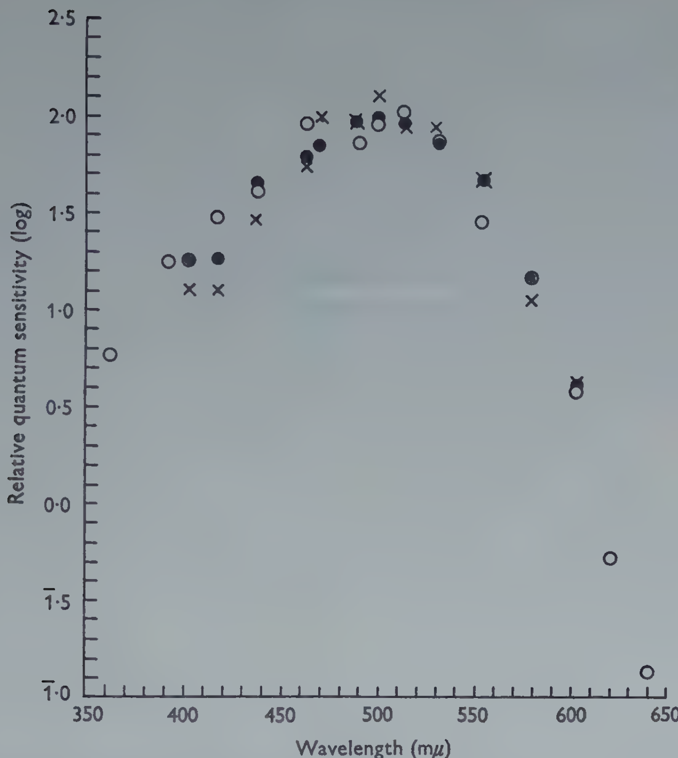

Fig. 3. The relative quantum sensitivity as a function of wavelength. The points (●) refer to *C. vomitoria* at level *A*, the circles (○) at level *B*. Crosses (×) refer to *C. erythrocephala* at level *A*. (Level *A* is equivalent to about 0·01 f.c., level *B* to 0·001 f.c.).

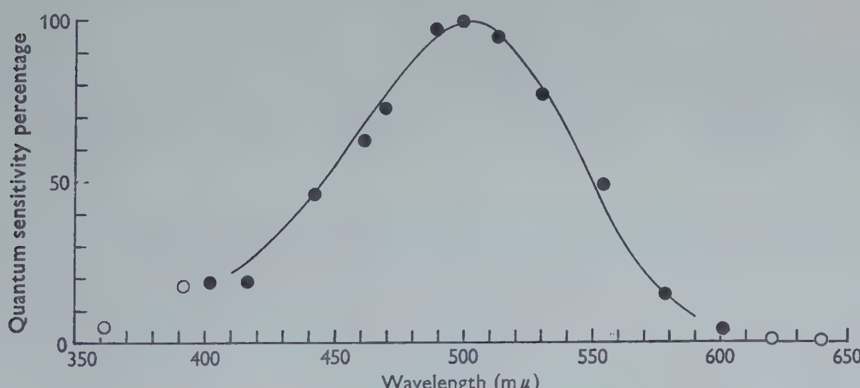

Fig. 4. The percentage quantum sensitivity for *C. vomitoria* as a function of wavelength. ● represents mean results at level *A*, ○ at level *B*. The line is the absorption curve for a hypothetical visual purple, with a maximum at 504 mμ (Dartnall, 1953).

In Fig. 5, the percentage of each group of maggots collected at the end of the bridge away from the light is plotted against the neutral density filter used. A blank experiment, in darkness, was included. The maggots were left for several hours and the experiment repeated. Both results are shown. The absolute threshold is about 4·0 log units

below level *A*, i.e. the maggots would just respond to a level of illumination of the order of 10^{-6} f.c., i.e. of about 10^{-5} lumens/m.^{2}. This is only one log unit more than the minimum intensity needed by man under dark-adapted conditions (Denton & Pirenne, 1954), with a natural pupil of about 0·5 cm.^{2} (Le Grand, 1948). Now, if man and maggot required the same quantity of light energy at threshold, the levels of illumination at the respective thresholds would be inversely proportional to the areas of the eye pupil in man, and of the total light-sensitive region of the maggot. The difference of one log unit, found experimentally, would on this basis lead to the conclusion that the light-sensitive region of the maggot was one-tenth of man's pupil area, i.e. 0·05 cm.^{2}. This is impossibly large, compared with the size of a maggot, and it is therefore more likely that the high sensitivity is due to some other cause, such as pigment concentration.

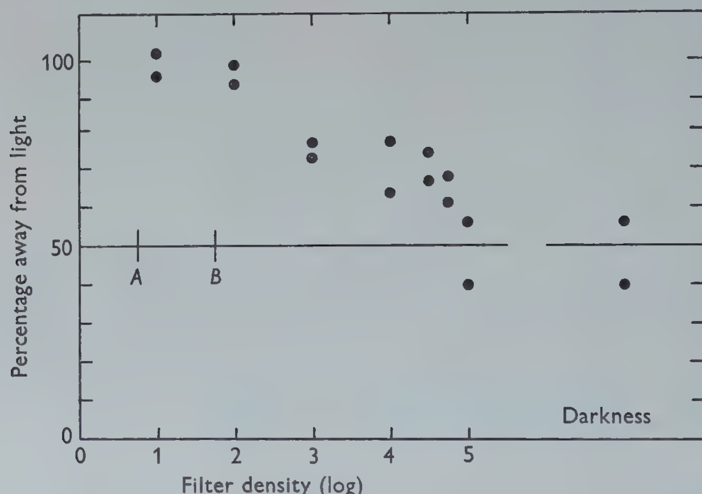

Fig. 5. Determination of the absolute threshold of *C. vomitoria* for 442 mμ; below this level, equal numbers of maggots travel towards the light and away from it. The intensity is governed by the neutral density filter shown in the abscissa. The levels of the relative thresholds are shown as *A* and *B*.

A direct determination of the author's own absolute threshold of vision, after 45 min. of dark adaptation, confirmed this remarkable efficiency of the maggot, area for area. An artificial pupil of area 2·5 mm.^{2} was set up at the position of the middle of the bridge, and neutral density filters were added until the standard light at level *A* was only just visible. At this level the intensity was only half a log unit less than at the absolute threshold for the maggot. Corrected to a pupil area of 0·5 cm.^{2}, this threshold is of the same order as that found by other workers.

DISCUSSION

Discussion of the method

Bolwig describes the trial-and-error progression of a maggot, which appears to be superimposed on a stronger or weaker drive to wander about at random.

Maggots nearer pupation respond more consistently to light than younger larvae.

For example, the same light which made 70% travel away, 2 days before pupation, caused 97% to travel away on the last day. In each case the maggots had rested undisturbed in the dark overnight. When maggots which had travelled to the light were gathered up, rested in the dark and tested separately, they divided in the same ratio as before; the group which had travelled away from the light did the same. This suggests that the photonegative behaviour is superimposed on random wandering, rather than that any of the maggots were photopositive.

The finding that older larvae are more responsive to light does not agree with Patten (1916). From a teleological point of view, it is in the interest of the maggots to leave the shelter of the food and disperse, before finding a dark corner to pupate. Bolwig attributes the mechanism of the more accurate orientation of older larvae, relative to the light, to the increasing shielding of the light-sensitive organs from light coming from behind; he says that younger larvae soon become tired and stop responding to light, as was also found in the present work.

The only effect of this lower response on the relative threshold determination is to make the graph of the percentage travelling away from the monochromatic light (e.g. Fig. 2) slope less steeply—it does not change the point of crossing the 50% line. Mast's work on blowfly maggots was a relative threshold, while Bolwig's threshold for the housefly maggot suffers the disadvantage of being an absolute threshold.

Certain precautions were taken with a knowledge of the habits of maggots. If they were damp from their food, they were mixed with dry bran before dark adaptation, since a smooth vertical surface presents no obstacle to a wet maggot. The bridge was cleaned with a damp cloth between experiments to remove the trails of the previous group, which maggots tend to follow, and the bridge was levelled with a spirit-level to ensure symmetry.

The cooling fan alone, without the lights, was found to have no effect.

Patten (1914) found that maggots of *C. erythrocephala* responded equally to a steady light and to a light interrupted 30 times a second, provided that the total quantity of light per second was the same. Since the Bunsen–Roscoe law is thus obeyed, it can make no difference whether 50 c./s. alternating or direct current is used for the incandescent stimulus lamps.

Comparison of present results with previous spectral sensitivity curves

In order to compare the present results for *Calliphora* larvae with the findings of other workers on muscid larvae and flies, the line of Fig. 5 is redrawn in each part of Figs. 6 and 7, extended to 362 and 640 mμ.

First, attention must be drawn to the simple nature of the maggot's spectral sensitivity curve—there is no sign of any maximum away from 504 mμ, either in the ultraviolet or red parts of the spectrum.

Where other authors give the percentage quantum sensitivity, or the data from which it may be calculated, this is shown in parts (*a*) to (*d*) of Fig. 6 and Fig. 7(*a*) to (*c*). Fig. 6(*a*), ●, shows the results of Mast (1917) for unidentified fleshfly maggots, at levels 7 and 20 m. candles, i.e. of the order of 1 f.c., for wavelengths between 422 and 625 mμ. The standard white light was at right angles to the monochromatic light, which was adjusted in intensity until the maggots set themselves midway between

the two. The maximum is at 504 mμ, the same as for the present work, but the curve is narrower.

Fig. 6(*a*), ○, is the absolute quantum sensitivity for the housefly maggot for wavelengths between 405 and 700 mμ, drawn from Bolwig (1946). The criterion here was the ability of maggots to re-orient themselves when the paper which they were on

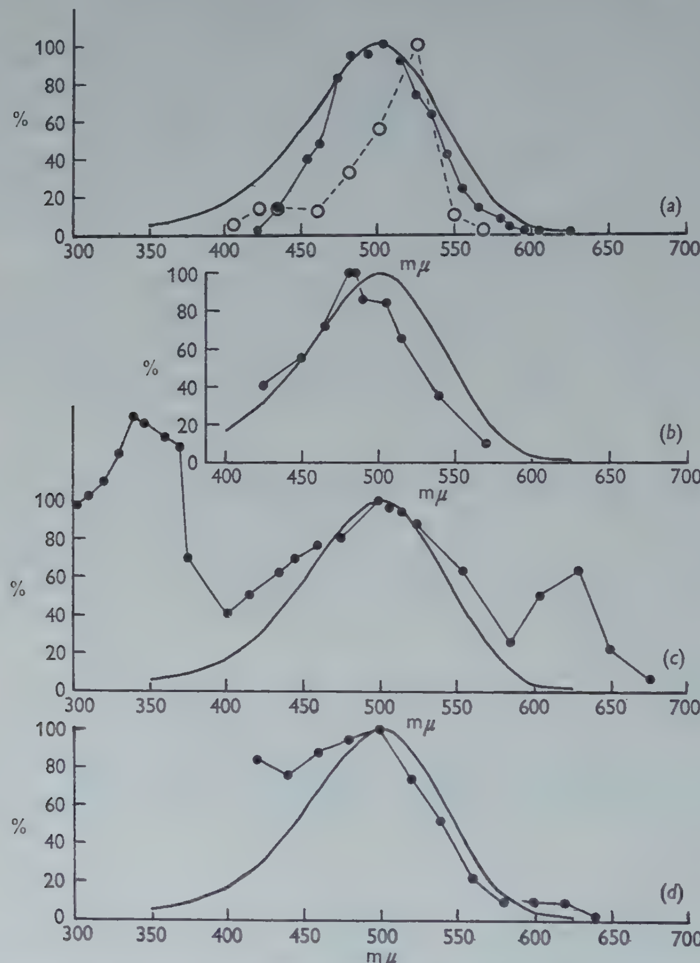

Fig. 6. Percentage quantum sensitivities found by other workers for muscid flies and larvae. The present results for *Calliphora* larvae are repeated in each diagram (thick line). (*a*) Maggots; ●, Mast, blowfly; ○, Bolwig, housefly. (*b*) Schneider, *C. erythrocephala*. Adult (optokinetic). (*c*) Walther & Dodt, *C. erythrocephala*. Adult (electrophysiological). (*d*) Donner & Kriszat. Adult fly (electrophysiological).

was turned through a right angle. The maggots were not all of the same age, described as 'second and third instar larvae', which may have influenced the absolute threshold method. (The peak at 520 mμ is sharp compared with other spectral sensitivity curves.) Again, there is no return of sensitivity at the red end of the spectrum; the ultra-violet was not investigated.

In Fig. 6(*b*) we come to the spectral sensitivity of adult flies, this time from

Schneider (1956), the only behavioural results for which we have the data in this form. This is an optokinetic method for *C. erythrocephala*, at a very low level of intensity, in the human scotopic region. The fly was glued to a glass capillary at the centre of a glass cylinder, outside which turned a concentric cylinder with vertical black stripes, illuminated by monochromatic light of wavelength between 425 and 570 mμ. When the fly had enough light to see the stripes, it attempted to follow them. The maximum sensitivity was at 480 mμ.

Walther & Dodt (1959) used an electrophysiological method, and their results are shown in Fig. 6(*c*), for wavelengths between 290 and 675 mμ. This curve is the mean result for 24 *C. erythrocephala* flies, all from the same stock. The criterion here was a constant height of on-effect of the electroretinogram (e.r.g.) in response to 40 msec. flashes of light every 30 sec., for the intact fly. These workers found that the relative height of the secondary maximum at 630 mμ was dependent on intensity, while the ultra-violet peak did not change with intensity in a regular way.

Donner & Kriszat (1949) also used the on-effect for the intact fly as an index of threshold between 440 and 640 mμ. The curve shown as Fig. 6(*a*) is for an individual fly which showed the peak at 500 mμ most clearly. For others the sensitivity was still rising at the violet end of their available spectrum. They found no apparent differences between the species *Musca domestica*, *Lucilia caesar*, *Calliphora vomitoria* and *Pollenia rudis*, and do not identify the fly to which this diagram refers. Adaptation increased the relative sensitivity to the violet end of the spectrum (420–40 mμ), the effect being greatest for green, then red, and then violet adapting lights.

Cameron (1939) found that houseflies were attracted by 366 mμ more than by any other wavelength. Weiss, Soraci & McCoy (1941), also using a behavioural method for houseflies, found a maximum of response to an equal energy spectrum at 490 mμ, a minimum at 460 mμ, and the response was still rising at 360 mμ. Granit (1947, p. 297) mentions an electrophysiological experiment, in which the eye of the housefly was found to be most sensitive to 490 mμ, sensitivity falling off rapidly on the long-wave side of the maximum. Granit extracted a 'very light-resistant carrot-coloured pigment' from the eye of the housefly. The only pigment bleached by light which has so far been found in a fly was extracted by Bowness & Wolken (1959) from housefly heads and had maximum absorption at 437 mμ.

The work of Autrum (1955), using the on-effect of the e.r.g. as an index of sensitivity, may provide a key to this diversity. Autrum uses the isolated head of *C. erythrocephala* and determines the magnitude of electrical responses at each wavelength for an equal-quantum spectrum. He gives the results at a series of quantum levels and, by replotting the response against quantum level, it is possible to find the relative quantum sensitivity at an arbitrary mV response level by interpolation. This has been done for three individual flies, at three levels of response, in increasing order, represented by ●, ○, and × in Fig. 7. Fig 7(*a*) and (*b*) refer to two wild-type *C. erythrocephala*, and 7(*c*) to a white-apricot mutant of the same species. In Fig. 7(*a*) the graph at the lowest intensity, ●, is of the same type as Fig. 6(*c*), and increasing intensity changes the wavelength of the peak around 500 mμ to about 530 mμ, at the same time increasing the relative height of a peak at about 630 mμ. 7(*b*) tells a similar tale, even more dramatically, as the peak in the red completely dwarfs the rest at the highest intensity.

Fig. 7(*c*) for the mutant form has only one maximum, around 515 mμ; and, again, the relative sensitivity at the shorter wavelengths decreases with increasing intensity.

It is clear that the same preparation can give more than one relative spectral sensitivity curve, according to intensity, and that there may be more than one 'Purkinje' type of sensitivity change.

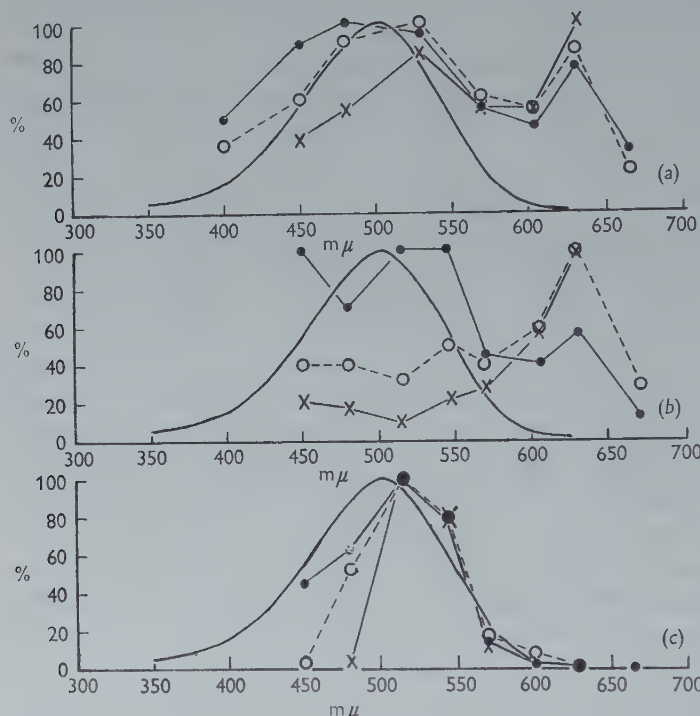

Fig. 7. Percentage quantum sensitivities for three adult *C. erythrocephala*, each at three levels of electrophysiological response, calculated from Autrum, 1955. The present results for *Calliphora* larvae are repeated in each diagram (thick line). (*a*) *C. erythrocephala* (wild). On effect: ●, 1 mV.; ○, 2 mV.; ×, 3 mV. (*b*) *C. erythrocephala* (wild). On effect: ●, 1 mV.; ○, 2 mV.; ×, 3 mV. (*c*) *C. erythrocephala* (white-apricot mutant). On effect: ●, 3 mV.; ○, 8 mV.; ×, 10 mV.

It seems possible that an electrode picks up the electrical activity of different types of nervous element from one preparation to another. (Though Walther & Dodt (1959) say that this effect is small compared with that found in the eye of the cockroach, *Periplaneta americana*.) Again, more than one retinal element may contribute, in different proportions. Autrum & Stumpf (1953) found that individual *C. erythrocephala* preparations, at high levels of illumination, gave response maxima at either 540 or 630 mμ, or else at both wavelengths. (In some cases there was evidence for wavelength discrimination, using a flicker method.)

The high sensitivity in the ultra-violet, maximal at 340 mμ (Walther & Dodt, 1959), varied from one specimen of *C. erythrocephala* to another (even between flies of the same stock and age) compared with the maximum near 500 mμ. Reducing the intensity of the stimulus always changed the relative sensitivity to red, but the ultra-violet sensitivity did not always change in the same direction.

Inspection of Figs. 6 and 7 shows that all these muscid flies have a maximum of sensitivity near 500 mμ which is especially prominent at low levels of illumination. This may well be a continuance from the visual mechanism of the larval state, most sensitive to 504 mμ. No light-sensitive pigment has yet been found to account for this; the atypical pigment found in the housefly by Bowness & Wolken (1959), with its maximum absorption at 437 mμ, is not a likely candidate. The form of the spectral sensitivity curve of the maggot would suggest a visual purple (or rhodopsin) and the low absolute threshold leads to the idea that such a pigment might be present in measurable concentration.

SUMMARY

1. The relative spectral sensitivity of larvae of *Calliphora vomitoria* and *C. erythrocephala* has been determined, using the maggot's natural tendency to travel towards the weaker of two opposing lights.

2. The response to light at each wavelength between 402 and 602 mμ was the same for the two species, within ±0·1 log unit, and the results are well fitted by the bell-shaped curve characteristic of a (hypothetical) visual purple, maximal at 504 mμ.

3. Reduction of the intensity of the white standard light by one log unit did not change the shape of the curve, and the extended wavelength range, between 362 and 640 mμ, shows no sign of a subsidiary maximum of sensitivity. The levels of illumination were 10^{-2} and 10^{-3} f.c., and the absolute threshold was found to be about 10^{-6} f.c.

4. The spectral sensitivity found here is compared with the results of other workers for muscid flies and larvae. It is concluded that one of the independent maxima of sensitivity found in the flies is a continuance from the larval state.

I should like to thank Prof. V. B. Wigglesworth for the *C. erythrocephala* culture used, and the many people who have helped me with their special knowledge and advice.

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